Role of the PD-1/PD-L1 Co-inhibitory Pathway in Immune-mediated Renal Injury

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von

Astrid Starke

aus Deutschland

Promotionskomitee

Prof. Dr. Roland Wenger (Vorsitz)
Prof. Dr. Rudolf P. Wüthrich (Leitung der Dissertation)
PD Dr. Ying Wäckerle-Men
Prof. Dr. Marcus Groettrup
Dr. Natividad Hernando

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ZUSAMMENFASSUNG

Immun-vermittelte Nierenerkrankungen wie etwa die tubulointerstitielle Nephritis bewirken eine tubuläre Dysfunktion und einen chronischen Nierenschaden bis hin zum terminalen Nierenversagen. Renale tubuläre Epithelzellen (TECs) sind das Hauptziel infiltrierender T-Zellen und interagieren mit diesen durch lösliche Faktoren und direkten Zell-Zell-Kontakt. TECs können als Antigen-präsentierende Zellen (APCs) fungieren, da sie MHC Klasse I und II Moleküle exprimieren sowie kostimulatorische Moleküle, welche unter inflammatorischen Bedingungen exprimiert werden.

Die hier dargestellten Untersuchungen zeigen, dass die Stimulation mit den Zytokinen IFN-β und IFN-γ die Expression der kostimulatorischen Moleküle ICOS-L, PD-L1 und CD40 auf TECs *in vitro* induziert, wohingegen die klassischen kostimulatorischen Moleküle B7.1 and B7.2 und andere kostimulatorische Moleküle nicht induziert werden. Die Behandlung mit dem Zytokin TGF-β erhöht die Expression von CD40 auf TECs, erniedrigt aber die Interferon-induzierte Expression von PD-L1. Verglichen mit professionellen APCs wie dendritischen Zellen (DCs) haben TECs eine eingeschränkte Expression von kostimulatorischen Molekülen, auch unter inflammatorischen Bedingungen. Demzufolge stimulieren TECs T-Zellen nur suboptimal, insbesondere im Vergleich mit DCs. PD-L1 exprimiert auf murinen TECs schützt diese partiell vor dem Angriff zytotoxischer OVA-Peptid-spezifischer T-Zellen. Zudem ist die Zytokinsekretion von OVA-spezifischen $CD4^+$ und $CD8^+$ T-Zellen *in vitro* reduziert, wenn sie mit TECs die eine hohe Expression von PD-L1 aufweisen, ko-kultiviert werden. In einem experimentellen Model der autoimmunen Nephritis, welches im transgenen RIP-mOVA Mausstamm etabliert wurde, ist PD-L1 auf TECs *in vivo* induziert. Demzufolge könnte PD-L1 auch in die Regulation von autoreaktiven T-Zell-Antworten *in vivo* involviert sein. Die PD-L1 Expression auf humanen TECs limitiert alloreaktive $CD4^+$ und $CD8^+$ T-Zell-Antworten *in vitro*. Die mRNA Expression von PD-1, PD-L1 und PD-L2 in humanen Biopsieproben von verschiedensten Transplantatsabstossungen lässt eine Beteiligung dieses koinhibitorischen Signalweges in humaner Nierentransplantation vermuten.

Es lässt sich schlussfolgern, dass PD-L1 exprimiert auf TECs autologe und

alloreaktive T-Zell-Antworten *in vitro* vermindert. Die Expression von PD-L1 auf renalen tubulären Epithelzellen könnte daran beteiligt sein, dass Ausmass der tubulointerstitiellen Nephritis zu kontrollieren. Ergebnisse dieser Studien erlauben die Hypothese zu formulieren, dass dieser Koinhibitorische Signalweg ein therapeutisches Ziel sein könnte, durch dessen Aktivierung immun-vermittelte Nierenerkrankungen behandelt werden könnten.

SUMMARY

Immune-mediated kidney diseases such as tubulointerstitial nephritis cause renal tubular dysfunction and chronic renal failure which may progress to end-stage renal disease. Renal tubular epithelial cells (TECs) are the main target of infiltrating T cells and interact with these cells through soluble factors and direct cell-cell contact. TECs can behave as antigen-presenting cells (APCs) due to their expression of MHC class I and II molecules as well as certain co-stimulatory molecules which are induced under inflammatory conditions.

The results presented here demonstrate that stimulation with cytokines, e.g. IFN-β and IFN-γ induces the expression of the co-stimulatory molecules ICOS-L, PD-L1 and CD40 on TECs *in vitro,* whereas the classical co-stimulatory molecules B7.1 and B7.2 and other known co-stimulatory molecules are not induced. The treatment with TGF-β increases CD40 on murine TECs but decreases the IFN-induced expression of PD-L1 *in vitro*. Thus, compared to professional APCs e.g. bone-marrow derived DCs (BM-DCs) TECs have a limited expression of co-stimulatory molecules even under inflammatory conditions. Consequently, TECs only induce suboptimal T cell responses *in vitro* when compared with BM-DCs. Nevertheless, PD-L1 on murine TECs partially protects them from the attack of OVA-peptide-specific $CD8^+$ T cells and reduces also the cytokine response of OVA-specific $CD8^+$ and $CD4^+$ T cells *in vitro*. PD-L1 may also be involved in regulating auto-reactive T cell responses *in vivo* since it is induced on TECs in an autoimmune model of tubulointerstitial nephritis established in RIP-mOVA mice. Furthermore, PD-L1 expression on human TECs limits allo-reactive $CD4^+$ and $CD8^+$ T cells responses *in vitro* and expression of PD-1, PD-L1 and PD-L2 in human biopsies of kidney transplant specimens suggests an involvement of this co-inhibitory pathway in human kidney transplantation.

In conclusion, PD-L1 on TECs limits auto- and allo-reactive T cell responses *in vitro* and expression of PD-L1 in murine and human kidneys on TECs might be involved in controlling the severity of tubulointerstitial nephritis. The findings of these studies allow to postulate a new hypotheses regarding the role of this co-inhibitory pathway as a possible therapeutic target to inhibit immune-mediated renal diseases upon activation of this pathway.

INTRODUCTION

The immune system is characterized by a perfect interplay between its innate and adaptive parts. This results in a specific defense against pathogens, and in a state of tolerance for the body's own structures. An imbalance triggering an impairment or an overshot reaction of the immune system can have fatal consequences. A diminution of the immune response leads to a dissemination of pathogens and may cause chronic infections whereas an overreaction results in a destruction of the body's own components which manifests as autoimmune disease.

1. The two signal model of T cell activation

T cells, powerful effectors of the adaptive immune system, recognize their antigen as a peptide bound on MHC (major histocompatibility complex) molecules expressed on antigen-presenting cells (APCs). In general, a full activation of a naïve T cell and thereby a differentiation into an effector cell requires two signals (Figure 1). The first signal is the antigen recognition delivered by the interaction of the T cell receptor (TCR) and its co-receptors CD4/CD8 with the specific peptide/MHC complex on APCs. $CD8^+$ T cells bind to MHC class I molecules and $CD4^+$ T cells to MHC class II molecules. The signal 1 alone is not efficient to induce proliferation and differentiation of T cells. A co-stimulation signal (signal 2) of the same APC is required to fully activate naïve T cells. In the absence of signal 2, T cells fail to respond effectively and are rendered anergic. Signal 2 is dependent on signal 1 because in the absence of the first signal, ligation of co-stimulatory receptors does not induce a T cell response.

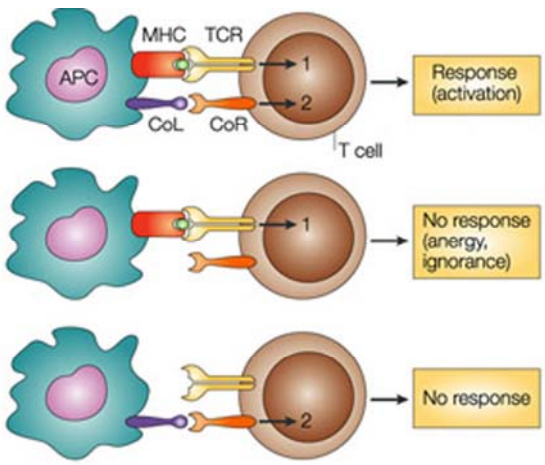

Figure 1: Two signal model of T cell activation
Taken from *Nat Rev Immunol 4:336-347, 2004* (1).

2. Co-stimulatory and co-inhibitory molecules

The second signal is delivered by co-stimulatory molecules expressed on APCs. Most co-signaling molecules are members of the immunoglobulin and tumor-necrosis factor (TNF) superfamilies (1). The classical co-stimulation pathway involves the immunoglobulin superfamily members B7.1 (CD80) and B7.2 (CD86) on APCs binding to their receptor CD28 that is constitutively expressed on T cells. The interaction among CD28 and the B7 molecules leads to an activation and the secretion of IL-2, enabling clonal expansion of T cells. CD28 is necessary and sufficient to induce optimal T cell activation, promotes viability and prevents anergy. Activated T cells then express a variety of molecules leading to a prolongation or modification of the co-stimulatory signal. For instance CD40L (CD154), a member of the TNF superfamily, is upregulated on T cells after signaling through CD28. It binds to the receptor CD40 on APCs which leads to a further activation of T cells as well as of APCs, resulting in a higher expression of B7 molecules on APCs and consequently in an amplification of T cell responses.

CTLA-4 (Cytotoxic T Lymphocyte Antigen-4, CD152) is another receptor that is induced on T cells 24-48 h after activation and is also ligated by B7 molecules but with a higher avidity than CD28. The interaction between CTLA-4 and B7 molecules results in an inhibitory signal delivered to naïve and primed T cells, making them less susceptible to the co-stimulation by APCs and reduces the production of IL-2 as well. Due to the ligation of CTLA-4 this co-inhibitory signal results in the termination of the clonal expansion of T cells (2-4).

Co-stimulatory and co-inhibitory molecules on APCs regulate the strength, course and duration of T cell responses. The balance of stimulatory and inhibitory signals is crucial to achieve protective immune responses while maintaining immunological tolerance and preventing autoimmunity. Due to this fine regulation of T cell responses co-stimulatory pathways are a target of therapeutic intervention. Prevention of acute rejection and optimal tolerance have been achieved in nonhuman primate models of kidney transplantation using anti-CD154 (anti-CD40L) (5). Therapeutic inhibition of the CD28 pathway is performed with the CTLA-4Ig molecules Abatacept and Belatacept that bind to B7 molecules on APCs such as dendritic cells (DCs), thereby

blocking their interaction with CD28 on T cells and inhibiting T cell activation and proliferation. Furthermore, CTLA-4Ig induces reverse signaling by binding to B7 molecules on DCs. This leads to an induction of the enzyme indoleamine 2,3-dioxygenase (IDO) which results in depletion of tryptophan and consequently in inhibition of T cell proliferation (1, 6). Abatacept is approved for the treatment of patients with rheumatoid arthritis and Belatacept is being investigated in clinical trials for renal transplantation (7).

Novel co-stimulatory and co-inhibitory molecules have been discovered during the last decade, including the new B7 family members PD-L1 and PD-L2, ICOS-L, B7-H3 and B7-H4 (Table 1). The PD-1/PD-L pathway will be discussed in detail in the next paragraph. The ICOS/ICOS-L pathway is important for stimulating effector T cell responses and T cell-dependent B cell responses, but is also involved in T cell tolerance (8). The role of B7-H3 in regulating T cell responses is contradictory. Some studies suggest a co-stimulatory and others a co-inhibitory function which may depend on different receptors for B7-H3 (9). B7-H4 is a negative regulator of T cell responses and expression of B7-H4 on tumors suggests a role in evasion of tumor immunity (8). Most of these molecules are constitutively expressed on professional APCs (DCs, macrophages, B cells) as well as on non-lymphoid organs, providing new insights into the regulation of T cell activation and tolerance in peripheral tissues.

Table 1: Overview of the B7 family members

Co-stimulatory/ Co-inhibitory molecule	Synonym	CD nomenclature	Binding partner
B7.1		CD80	CD28, CTLA-4, PD-L1
B7.2		CD86	CD28, CTLA-4
B7-H2/B7h	ICOS-L, B7RP-1	CD275	ICOS
B7-H1	PD-L1	CD274	PD-1, B7.1
B7-DC	PD-L2	CD273	PD-1
B7-H3		CD276	TLT-2, n.d.
B7-H4	B7x, B7S1, VTCN1	?	BTLA-4

CTLA-4: Cytotoxic T lymphocyte attenuator; PD-1: Programmed Death 1; ICOS: Inducible Costimulator; TLT-2: (TREM)-like transcript 2; VTCN1: V-set domain containing T cell activation inhibitor 1; BTLA-4: B and T lymphocyte associated protein 4; n.d. not determind

3. The PD-1/PD-L pathways - their expression and function

The Programmed Death 1 (PD-1, also called CD279) molecule and its ligands PD-L1 (also named B7-H1 and CD274) and PD-L2 (also called B7-DC and CD273) belong to the B7/CD28 family and are important pathways delivering inhibitory signals to T cells. PD-1 has been discovered in 1992 in a T cell hybridoma undergoing cell death (10). The ligands PD-L1 and PD-L2 have been identified nearly 10 years later in 2000 and 2001, respectively (11, 12).

3.1. Distribution and structure of PD-1, PD-L1 and PD-L2

PD-1 expression has been found on T and B cells, NK cells, activated monocytes and DCs. PD-1 is weakly expressed on resting T cells but is induced after ligation of the TCR. PD-1 is encoded by the Pdcd1 gene on chromosome 1 in mice and on chromosome 2 in humans. It is a 50-55 kDa type I monomeric receptor exhibiting one immunoglobulin variable (IgV)-like domain and an immunoglobulin constant (IgC)-like domain in the extracellular portion, a transmembrane region and an intracellular domain composed of an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine switched motif (ITSM). Engagement of PD-1 leads to phosphorylation of its two intracellular tyrosines, followed by a recruitment of the phosphatase SHP-2 (Src homology 2-domain-containing tyrosine phosphatase) to the ITIM and ITSM motifs. The activation of the phosphatase blocks the signaling of the PI3K pathways and downstream of Akt resulting in inhibition of early antigen receptor (TCR, BCR) signaling (Figure 2). Therefore, PD-1 needs to be in proximity to the antigen receptor. Ligation of PD-1 on T cells by its two ligands decreases the induction of certain cytokines including IFN-γ, TNF-α and IL-2 as well as the cell survival factor Bcl-xL. This reduction can be overcome by co-stimulation through CD28 or IL-2 (Figure 2). PD-1 typically has greater effects on cytokine production than on cellular proliferation (1, 13).

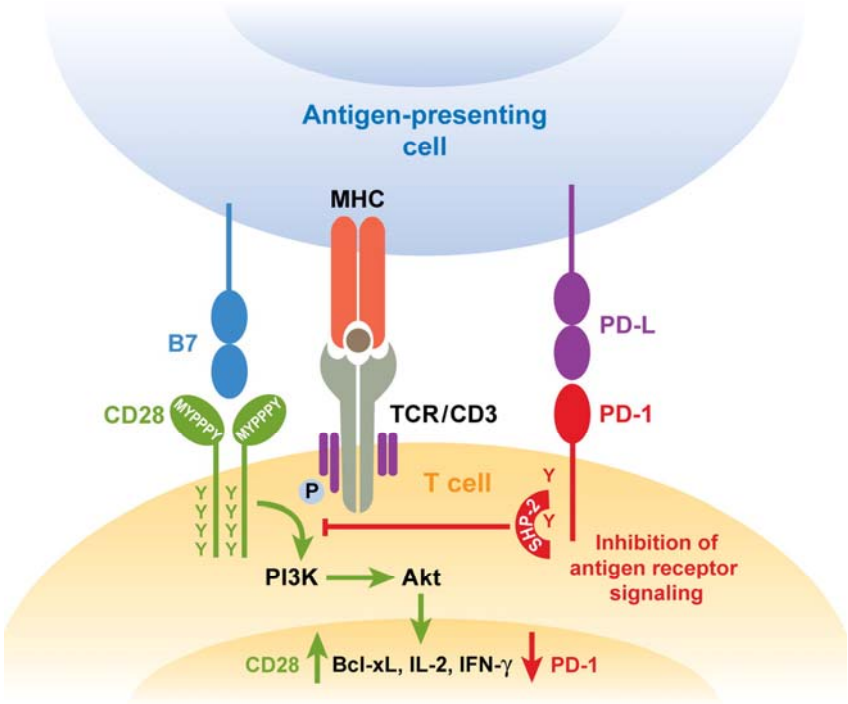

Figure 2: Ligation of PD-1 on T cells inhibits TCR signaling. Taken from *Ann Rev Immunol 26: 677-704, 2008* (13).

PD-L1 is a membrane protein which consists of one IgV-like domain and one IgC-like domain, a transmembrane part and a short intracellular domain. PD-L1 is encoded by the Cd274 gene on mouse chromosome 19 and on chromosome 9 of humans. PD-L1 is constitutively expressed on mouse T and B cells, DCs, macrophages, mesenchymal stem cells and can be found on a variety of nonhematopoetic and parenchymal cells including lung, liver, heart, brain, placenta and kidney. PD-L1 is upregulated on activated T and B cells and on a wide range of cells after stimulation with type I and II interferons (11, 13).

PD-L2 is also a transmembrane protein exhibiting the same structure as PD-L1 but different lengths of the cytoplasmic domain have been reported. PD-L2 is encoded by the Pdcd1lg2 gene adjacent to Cd274 but its expression is much more restricted compared to the PD-L1 expression. PD-L2 can be induced on DCs, monocytes and macrophages and it is also found on lung, vascular endothelial cells and placenta (12, 13). The distinct expression pattern of PD-L1 and PD-L2 suggest broad roles for PD-L1 in peripheral organs and selective roles for PD-L2 in lymphoid organs.

3.2. The role of the PD-1/PD-L1 pathway in tolerance and autoimmunity

An inhibitory role on the immune system of the PD-1/PD-L pathway on T cell activation has first been identified in Pdcd1$^{-/-}$ C57BL/6 and Pdcd1$^{-/-}$ Balb/c mice that develop mild glomerulonephritis and dilated cardiomyopathy, respectively (14, 15). These findings have suggested that the PD-1/PD-L pathway has a critical role in the induction and maintenance of tolerance and therefore in the control of autoimmunity. PD-L1 and PD-L2 expressed in the thymus interact with PD-1 expressing immature thymocytes and influence the positive and negative selection of T cells, thereby contributing to central tolerance (13).

The broad expression of PD-L1 on parenchymal tissues and immune-privileged sites such as placenta and eyes has further suggested an involvement in the regulation of peripheral tolerance. Sharpe et al. propose the following model (Figure 3) how the PD-1/PD-L1 pathway may control peripheral tolerance and autoimmunity:

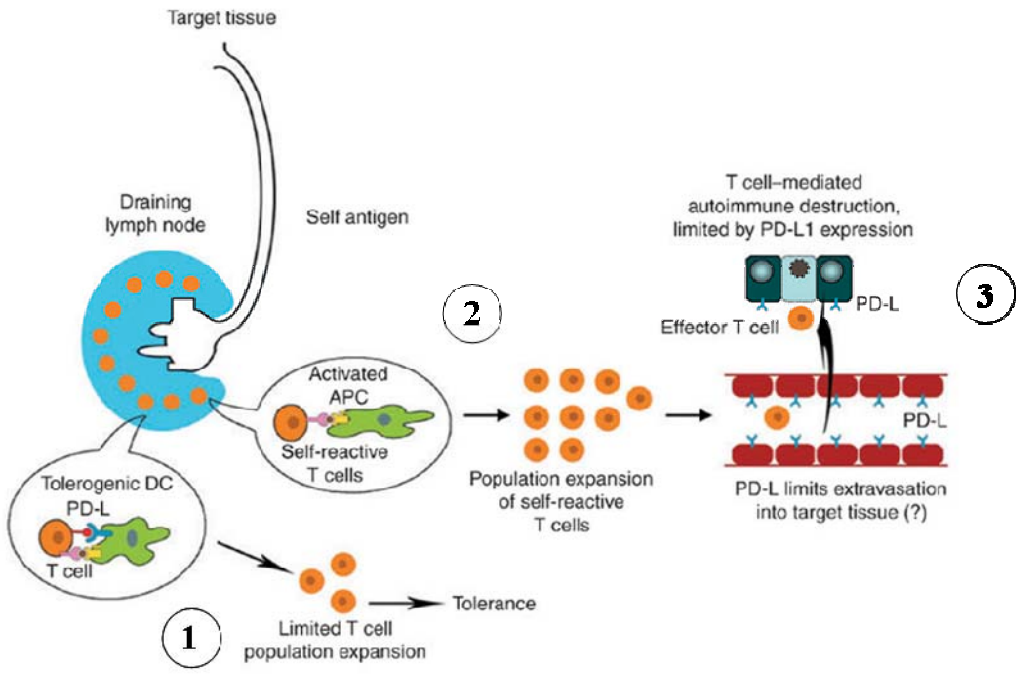

Figure 3: Control of autoimmunity by the PD-1/PD-L pathway. Adapted from *Nat Immunol 8: 239-245, 2007 (16).*

1) The presentation of self-antigens by immature DCs expressing PD-L1/L2 limits the expansion of self-reactive T cells and induces tolerance. In contrast, 2) activated DCs

may trigger the proliferation of self-reactive T cells which then home to the tissue expressing the self-antigen. 3) The extravasation of the T cells may be limited by PD-L1 expression on vascular endothelial cells. Furthermore, the expression of PD-L1 may protect the tissue from the attack of self-reactive T cells whereas tissue cells that do not express PD-L1 are destroyed (16).

Various studies support the critical role of the PD-1/PD-L pathway in regulating tolerance. Expression of PD-L1 in the placenta increases at the beginning of the second trimester and promotes fetal-maternal tolerance. The inhibition of PD-L1 with antibodies has increased the abortion rate of allogeneic fetuses and has been associated with increased T cell infiltration into the placenta (17, 18). Intestinal tolerance to self-antigens can be converted to autoimmune enteritis when the PD-1/PD-L pathway is blocked (19). Animal models of autoimmune diseases including diabetes and experimental autoimmune encephalomyelitis (EAE) have been exacerbated after blockade of the PD-1/PD-L1 pathway (20-22). A role for the PD-1 pathway in human autoimmune diseases has been suggested by polymorphisms in the Pdcd1 gene that have been associated with systemic lupus erythematosus (SLE), type 1 diabetes, rheumatoid arthritis, Grave's disease and multiple sclerosis (23).

Allograft tolerance is also partially mediated by PD-1/PD-L interactions (24, 25). PD-1 and PD-L1 are upregulated on allo-reactive T cells. Blockade of PD-L1, but not PD-L2, has accelerated rejection of MHC class II mismatched skin grafts in mice (26). This has also been shown for experimental models of heart, corneal and pancreas transplantation (27-29). Graft-versus-host disease (GVHD) lethality is accelerated by an IFN-γ-dependent mechanism in a murine model of acute GVHD when PD-1 engagement is prevented (30). It has been demonstrated in human liver transplantation that graft-versus-host-reactive $CD8^+$ T cells express high levels of PD-1 and that blockade of PD-L1 on host-derived cells enhances the allo-reactivity of these $CD8^+$ T cells *in vitro* (31).

A functional PD-1/PD-L pathway is beneficial in limiting expansion of self-and allo-reactive T cells and thereby prevents autoimmunity and allograft rejection. On the other hand increased expression of this pathway has also been found in many human cancers and infectious diseases with detrimental effect.

3.3. The role of the PD-1/PD-L1 pathway in tumor escape and infections

Tumor cells escape the immune response by expressing PD-1 ligands. An increased expression of PD-L1 has been found on human cancer tissues of lung, ovary, bladder, breast, stomach, colon, pancreas, liver, urothelial cells and kidney. For many of them, a strong correlation of PD-L1 expression on tumor cells has been associated with a poor prognosis (32-34). Thompson et al. have reported that patients with high tumor and/or lymphocyte PD-L1 are 4.5 times more likely to die from renal cell carcinoma than patients exhibiting low levels of PD-L1. Furthermore, the expression of PD-1 on tumor infiltrating immune cells has also been associated with poor outcome in these patients suffering from renal cell carcinoma (35, 36). A new mechanism has linked the loss of the tumor suppressor PTEN (phosphatase and tensin homolog) with a posttranscriptional increase of PD-L1 in human glioma. Glioma cell lines that do not express PTEN have been resistant to the cytolytic activity of tumor-specific T cells and the blockade of PD-1 partially restored the lytic activity (32, 37).

PD-1 and its ligands also have important roles in regulating immune defenses against pathogens that cause acute and chronic infections. $Pdcd1^{-/-}$ mice clear an adenovirus infection more rapidly but develop more severe hepatocellular injury than wild type mice suggesting that the PD-1/PD-L pathway limits the detrimental consequences of overshooting virus-specific effector T cells (13, 38). Viruses that cause chronic infections are using the PD-1/PD-L pathway to evade the immune system (13). Barber et al. have shown that $CD8^+$ T cells of mice that have been chronically infected with LCMV expressed high levels of PD-1 and have been exhausted. Blockade of PD-1 and PD-L1 *in vivo* has reduced the viral load due to a restored ability of $CD8^+$ T cells to proliferate, secrete cytokines and kill infected cells (39). $CD8^+$ T cell exhaustion due to PD-1 expression has also been demonstrated in human chronic viral infections including HIV, HBV and HCV and has even been associated with disease progression (40-43). A key role for the PD-1/PD-L pathway has also been shown for chronic bacterial infections like Helicobacter pylori as well as for parasitic infections in mice (13).

3.4. Reverse signaling of PD-L1

Some of the studies have demonstrated that blockade of PD-L1 has not the same effect as inhibition of PD-1, suggesting the existence of another receptor for PD-L1. Recently, B7.1 has been identified as another binding partner for PD-L1 identifying reverse signaling through PD-L1 and B7.1 (13, 44, 45). Both can interact through their IgV-like domains. B7.1/PD-L1 specific interactions can induce inhibitory signals for T cells. The ligation of PD-L1 on $CD4^+$ T cells by B7.1 or ligation of B7.1 on $CD4^+$ T cells by PD-L1 delivers an inhibitory signal in terms of decreased proliferation and impaired cytokine production. PD-L1 and B7.1 are both expressed on T and B cells, DCs and macrophages suggesting a bidirectional interaction between B7.1 and PD-L1. PD-L1 on non-hematopoietic cells may interact with PD-1 as well as with B7.1 on T cells. Reverse signaling of PD-L1 has been also shown on tumor cells receiving survival signals after engagement of PD-L1 by PD-1 expressing immune cells (46).

3.5. Therapeutic modulation of the PD-1/PD-L1 pathway

The broad involvement of the PD-1/PD-L pathways in regulating immune responses provides therapeutic opportunities. PD-1/PD-L antagonists may be beneficial in augmenting anti-microbial and anti-tumor immunity (13, 32). A fully humanized anti-PD-1 monoclonal antibody has been developed and is in Phase 1 clinical trials for cancer. Thus far this antibody has been shown to be safe and well tolerated in patients with advanced hematologic malignancies (47). Furthermore, blockade of PD-1 has enhanced the expansion and functional capacity of human melanoma antigen-specific cytotoxic T cells *in vitro* (48). PD-1/PD-L agonists that suppress adverse immune responses may be useful to treat autoimmune diseases and transplant rejection. Enhancement of the PD-1/PD-L pathway in humans could also be achieved by treatment with IFN-β that is used for the treatment of multiple sclerosis (MS) and has induced expression of PD-L1 and PD-L2 on monocytes and $CD4^+$ T cells of patients with MS (49, 50).

4. Immune-mediated tubulointerstitial renal diseases

Immune-mediated tubulointerstitial renal diseases represent an important but heterogeneous class of kidney pathologies. Typical diseases include primary acute and chronic tubulointerstitial nephritis (TIN), and secondary interstitial nephritides which are seen in the context of systemic diseases such as sarcoidosis, Wegener's disease, systemic lupus erythematosus (SLE), or Sjögren's syndrome.

Primary acute TIN is often due to "allergic" immune reactions against drugs, mostly antibiotics, diuretics and non-steroidal anti-inflammatory drugs, or it is caused by infections with bacteria or viruses including streptococci, staphylococci, EBV or HIV (51). Furthermore, the main feature of acute cellular transplant rejection represents tubulitis (Figure 4A) – the invasion of the tubular epithelium by infiltrating T cells and macrophages – which often resembles the histopathological picture of primary TIN (52).

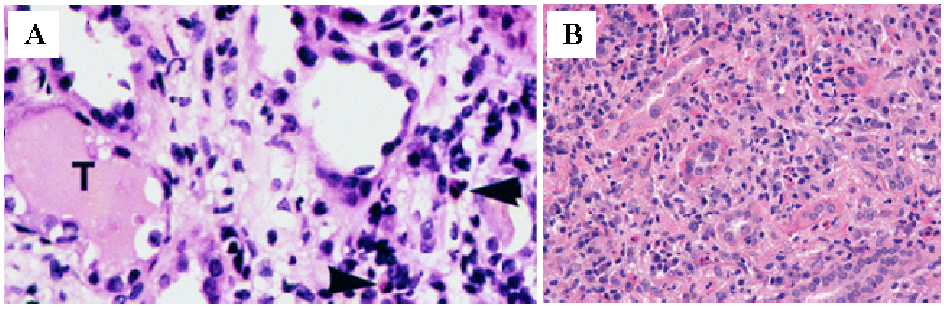

Figure 4: (A) Renal biopsy of a patient with acute allergic interstitial nephritis secondary to ingestion of non-steroidal inflammatory drugs. There is interstitial edema and a mixed cellular infiltrate which contains mononuclear cells, leukocytes and eosinophils (arrowheads). There is also evidence of tubulitis (T) with foci of tubular epithelial cell necrosis (orig. magnification x300, H&E stain). Picture and legend taken from *Kidney Int 54:313-327, 1998* (51). **(B)** Chronic interstitial nephritis shows interstitial fibrosis and a mixed inflammatory infiltrate composed of lymphocytes, plasma cells, and eosinophils (orig. magnification x28.). Picture and legend taken from *Am J Kidney Dis 49: E7-E10, 2007* (53).

In acute interstitial nephritis, the tubular damage leads to renal tubular dysfunction, with or without renal failure. Regardless of the severity of the damage to the tubular epithelium, the renal dysfunction is generally reversible. Conversely, chronic tubulointerstitial nephritis is characterized by interstitial scarring, fibrosis (Figure 4 B), and tubular atrophy, resulting in progressive chronic renal failure and end-stage

renal disease.

Cellular immunity is the principal effector mechanism of tubulointerstitial renal injury. Various types of immune cells including neutrophils, eosinophils, macrophages, DCs, B cells, NK cells and activated T cells are found in the tubulointerstitial compartment of patients with TIN and renal allograft rejection (RAR). These cells contribute extensively to tissue damage and progressive renal failure *via* their respective intrinsic mechanisms.

Activated T cells are the predominant effector cells in tubulointerstitial renal injury (54, 55). Infiltrating $CD4^+$ and $CD8^+$ T cells are found in abundance in most tubulointerstitial disease processes, including TIN and RAR, especially in acute T cell-mediated rejection. Their functional role in nephritic inflammation may be exerted via interaction with other infiltrating and/or resident cells such as macrophages and DCs and/or via their direct contact with renal tubular epithelial cells (TECs) which present auto- or foreign antigens to specific T cells. For example, in biopsies of drug-induced acute interstitial nephritis drug-specific T cells were found in the T cell infiltrate, suggesting that the drug was presented in the kidney in an immunogenic way, thereby triggering an immune response or reactivating immigrating cells (56).

5. Renal TECs are "non-professional" APCs

Renal proximal tubular epithelial cells (TECs) are the main target of infiltrating leukocytes during immune-mediated kidney diseases. There is an intensive cross-talk between resident TECs and infiltrating cells, either by soluble factors or by direct contact. TECs can be directly involved in renal parenchymal immune responses by acting as APCs and target cells of T cells (Figure 5).

After stimulation with leukocyte-derived cytokines e.g. IL-1, IL-17, TNF-α or IFN-γ, TECs become activated and release a broad variety of cytokines and chemokines, including IL-6, IL-15, IL-18, TNF-α, TGF-β, IL-8, MCP-1, MIP-1α and RANTES. This has been shown in cell culture experiments but also in renal graft biopsies with rejection (57). External stress factors e.g. protein overload, active peptides or ischemia also trigger cytokine and chemokine production by TECs. The cytokine secretion by TECs influences T cells and monocytes in terms of proliferation, survival and serves as chemoattractant to recruit further leukocytes. The cytokines also affect TECs in an autocrine manner, for example TGF-β can lead to transdifferentiation of TECs into myofibroblasts and overproduction of matrix components by TECs results in fibrosis and consequently in loss of kidney or graft function (58).

As a response to immune stimuli, TECs upregulate the expression of MHC molecules, adhesion molecules, co-stimulatory and co-inhibitory molecules favoring a direct contact with leukocytes and modulating T cell responses. Like most cells of the body, TECs constitutively express MHC class I molecules but stimulation with IL-1, TNF-α and IFN-γ further increases MHC class I molecules and also induces MHC class II expression (59, 60). Therefore, TECs can behave as APCs presenting peptides of self- and foreign origin to $CD4^+$ and $CD8^+$ T cells. Adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen-3 (LFA-3), vascular cell adhesion molecule 1 (VCAM-1) are also induced on TECs after cytokine stimulation and can stabilize the interaction among TECs and lymphocytes. The classical co-stimulatory molecules B7.1 and B7.2 are not expressed on TEC and can not be induced upon stimulation with IFN-γ, TNF-α or LPS suggesting that TECs are unable to fully activate T cells. However, treatment with IL-17, IFN-β, IFN-γ and TNF-α enhances CD40 expression on TECs *in vitro* indicating

an involvement of the CD40-CD40L pathway in the interaction between TECs and T cells (57). The expression of ICOS-L has been found in normal human kidneys and in renal allograft biopsies with rejection and interstitial nephritis, particularly in the areas of mononuclear cell infiltration suggesting a regulation of T cell activation by the ICOS/ICOS-L pathway. ICOS-L can be upregulated by interferon treatment on human and murine TECs *in vitro* and inhibits T cell responses (61, 62) in terms of IL-2 and IFN-γ production. In a mouse model of kidney allotransplantation, the blockade of ICOS has resulted in continuous inflammatory processes with progressive tissue damage and graft failure (63). Thus, this pathway may be one of the mechanism to inhibit immune-mediated TEC injury (57, 64).

PD-L1 and PD-L2 expression has also been found on human and murine TECs, especially after IFN-γ stimulation *in vitro* (65, 66). In a mouse model of renal allografts, PD-L1 is expressed on proximal and distal tubules in rejected transplants whereas staining of normal kidneys and isografts is negative. Furthermore, PD-L1 on TECs has inhibited IL-2, IL-4 and IFN-γ production of $CD4^+$ T cells in TEC-T cell co-culture experiments (67). These data suggest that PD-L1 on TECs is also involved in regulating immune responses in the kidney.

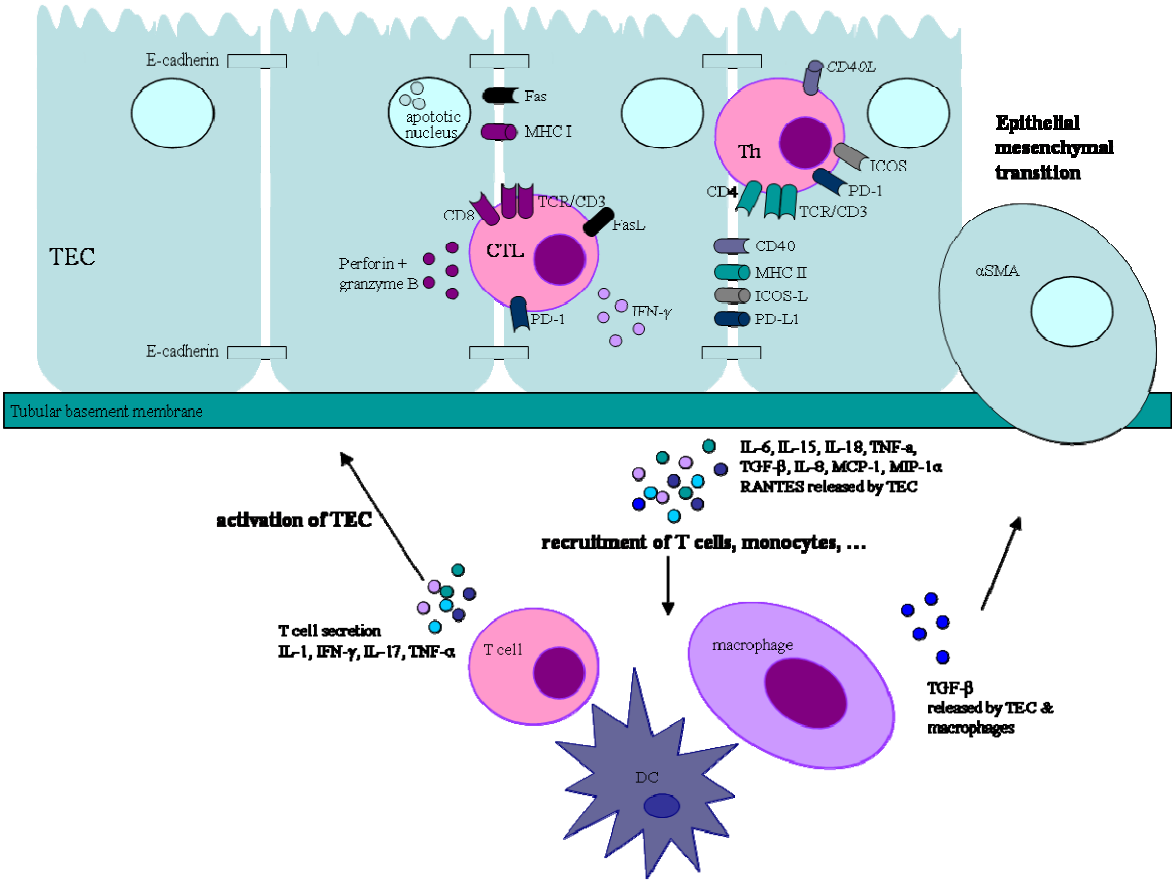

Figure 5: Complex interaction between TECs and infiltrating immune cells. Activated T cells are attracted into cortical tubules probably via chemokines from tubular cells, made in response to cytokines from inflammatory cells. T cells enter between TECs (tubulitis) and cytotoxic T cells may cause apoptosis by releasing cytolytic granules containing granzymes and perforin or by FasL/Fas interaction. IFN-γ secretion by $CD8^+$ and $CD4^+$ T cells may induce expression of MHC class I and II, CD40, ICOS-L and PD-L1 on TECs and may modulate T cell responses. Chronic exposure of TECs to TGF-β released by macrophages or TECs themselves may lead to epithelial-mesenchymal transition of TECs. These cells are characterized by α-smooth muscle actin (αSMA) and loss of E-cadherin expression and may infiltrate the interstitium and contribute to fibrosis. Resident/infiltrated DCs or macrophages may also interact with infiltrating T cells as well as with TECs either directly or by soluble mediators.
Figure and legend were modified from *Annu.Rev.Pathol. 3:189-220,2008* (52)

PURPOSE

The role of the PD-1/PD-L pathway in immune-mediated renal diseases has not been described yet. First studies have shown that PD-L1 is expressed on renal tubular epithelial cells *in vitro* and *in vivo* and therefore suggest an involvement in the regulation of immune responses in the kidney. Whether PD-L1 and/or PD-L2 on renal tubular epithelial cells can regulate $CD4^+$ and $CD8^+$ T cell responses is largely unknown.

The aim of this dissertation has been a further description and identification of the role of the PD-1/PD-L pathway on renal tubular epithelial cells in regulating T cell responses. The interaction between TECs and T cells has been investigated using *in vitro* co-culture experiments of TECs and T cells of both mouse and human origin. Furthermore, an experimental model of tubulointerstitial nephritis has been established to identify the *in vivo* relevance of PD-L1 on renal tubular epithelial cells.

CHAPTER 1

Limited co-stimulatory molecule expression on renal tubular epithelial cells impairs T cell activation

Ying Waeckerle-Men, **Astrid Starke**, Patricia R. Wahl and
Rudolf P. Wüthrich

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Limited Costimulatory Molecule Expression on Renal Tubular Epithelial Cells Impairs T Cell Activation

Ying Waeckerle-Men[a] Astrid Starke[a] Patricia R. Wahl[a] Rudolf P. Wüthrich[a, b]

[a]Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zürich-Irchel, and
[b]Clinic for Nephrology, University Hospital Zürich, Zürich, Switzerland

Key Words
Renal proximal tubular epithelial cell · T cell activation · Inflammatory cytokine · Costimulatory molecules · PD-L1

Abstract
Background/Aims: MHC molecules are upregulated on renal proximal tubular epithelial cells (TEC) under inflammatory conditions. This allows TEC to act as 'non-professional' antigen-presenting cells (APC). The aim of this study was to compare the costimulatory molecule expression pattern and the T cell activation capacity between renal TEC and professional APC, e.g. bone marrow-derived dendritic cells (BM-DC). ***Methods:*** Flow cytometry analysis was used to study the costimulatory molecule surface expression on TEC or BM-DC. Ovalbumin-specific CD4 and CD8 T cell activation induced by TEC or BM-DC was compared, in terms of T cell proliferation, cytokine production and CTL activity. ***Results:*** TEC did not constitutively express significant amounts of costimulatory molecules. Stimulation of TEC with IFN-β or IFN-γ, but not other tested cytokines, enhanced the expression of PD-L1, ICOS-L and CD40. Compared to BM-DC, TEC only induced suboptimal T cell activation. Blockade of PD-L1 on both APC strongly increased T cell activity. Furthermore, high PD-L1-expressing TEC were more resistant to the cytolysis by CTL. ***Conclusion:*** The low costimulatory molecule expression may explain the suboptimal T cell activation by TEC. The IFN-upregulated negative costimulatory molecule PD-L1 on TEC may play a protective role to limit tissue injury during renal parenchymal immune responses.

Introduction

Renal proximal tubular epithelial cells (TEC) are a major target of renal damage during immune-mediated kidney diseases. Under nephritic conditions, inflammatory cytokines are produced either by infiltrating immune cells or by activated TEC themselves. Intrarenal inflammatory cytokine stimulation promotes the upregulation of MHC class I and class II molecules on the surface of renal TEC, allowing TEC to act as 'non-professional' antigen-presenting cells (APC) to activate antigen-specific CD4 and CD8 T cells [1–3]. As a consequence, TEC may become direct targets of CD8 cytotoxic T lymphocytes (CTL) causing tubular damage and loss of renal function.

It has been well established that in addition to the first signal (the binding between MHC/peptide complex and T cell receptor), costimulatory or coinhibitory molecules provide the second signal that is essential in determining the magnitude of T cell activation or T cell tolerance [4, 5]. Besides the well-known classical costimulatory pathways B7/CD28, CD40/CD40L and the coinhibitory pathway B7/CTLA-4, several novel costimulatory molecules have been discovered during the last decade, including new B7 family members PD-L1 and PD-L2 (ligands 1 and 2 for the programmed death-1, PD-1), also known as B7-H1 and B7-DC, respectively, ICOS-L (the ligand for the inducible costimulator, ICOS), also known as B7RP-1, B7-H3, B7-H4 and new TNFR family members OX40L, 4-1BBL, CD70 and CD30L [4, 5]. Most of these molecules are constitu-

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Dr. Y. Waeckerle-Men
Institute of Physiology, University of Zürich
Winterthurerstrasse 190
CH–8057 Zürich (Switzerland)
Tel. +41 44 635 5107, Fax +41 44 635 6814, E-Mail ying.waeckerle-men@access.uzh.ch

tively expressed on professional APC such as macrophages and dendritic cells and can be further upregulated when stimulated with pro-inflammatory cytokines. These novel costimulatory/coinhibitory molecules may provide an alternative secondary signal for the control of T cell responses under immunopathologic conditions and for the maintenance of peripheral tolerance [4–7].

A prominent upregulation of the costimulatory molecules PD-L1 and ICOS-L on renal TEC with IFN-γ stimulation has been described by our group and others [8–11]. Recently, we observed that the type I IFN, IFN-β, was also able to induce PD-L1 upregulation on renal TEC [12]. However, a full understanding of the expression pattern of costimulatory molecules on renal TEC and/or their response profile to inflammatory cytokines has not yet been elucidated. Furthermore, how the costimulatory signal (signal 2) influences or impacts the T cell activation capacity of TEC is not fully clear so far. In this study, we compared the expression profile of all known B7 and TNFR family costimulatory molecules under non-inflammatory conditions between murine renal TEC primary cultures and bone marrow-derived dendritic cells (BM-DC). We also examined the costimulatory molecule expression on TEC upon stimulation with a large range of selected inflammatory cytokines which can be detected in the inflamed kidney. The capacity of presenting MHC class I- and class II-restricted ovalbumin (OVA) antigens by renal TEC was then investigated and compared with professional APC, e.g. BM-DC.

Materials and Methods

General Reagents

Cell culture reagents were obtained from Invitrogen (Gaithersburg, Md., USA) and Sigma (St. Louis, Mo., USA). All recombinant mouse cytokines used in this study were purchased from R&D Systems (Oxford, UK). All anti-mouse monoclonal antibodies (mAbs) were bought from eBioscience (San Diego, Calif., USA) if not specified. Chicken OVA protein was purchased from Sigma and the H-2K^b-restricted OVA peptide 257–264 ($OVA_{257–264}$) was received from Proimmune (London, UK). An H-2K^b-restricted β-galactosidase peptide 497–504 (β-$Gal_{497–504}$) was used as a negative control. Mouse CD8a (Ly-2), CD4 (L3T4) and CD11c (N418) MACS microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Animals

TCR transgenic OT-1 mice [13] were obtained from Jackson Laboratories (Bar Harbor, Me., USA). C57BL/6 (B6) mice were purchased from Harlan (Horst, Netherlands). All animals were used at 8–16 weeks of age. The study protocol was approved by the regulatory commission for animal studies of the Canton of Zurich, Switzerland.

Generation of Primary Renal TEC, BM-DC

Primary cultures of murine renal proximal TEC were prepared and cultured as described previously [14]. BM-DC were prepared and matured with lipopolysaccharide (LPS, Sigma, 10 μg/ml) for 2 days as described elsewhere [15]. The matured BM-DC were purified using mouse CD11c MACS microbeads before being used in experiments. All cells were maintained at 37°C with 5% CO_2.

Isolation and Activation of OVA-Specific OT-1 CD8 T Cells

H-2K^b-restricted $OVA_{257–264}$-specific CD8 OT-1 cells were isolated from spleen and lymph nodes of naïve OT-1 mice with mouse CD8a MACS microbeads. The freshly isolated OT-1 CD8 T cells were either used immediately for antigen presentation assays, or activated in vitro using B6 splenocytes pulsed with 0.1 μg/ml of $OVA_{257–264}$ peptide and incubated in T cell medium (DMEM supplemented with 5 μM 2-mercaptoethanol, 1% HEPES, 1,000 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS). After 5–6 days of stimulation, activated OT-1 CTL were used for CTL assays.

Immunization

To obtain OVA protein-specific CD4 T cells, B6 mice were immunized subcutaneously with 100 μg of OVA protein in PBS emulsified in Incomplete Freund's Adjuvant (IFA) at the base of the tail. Two weeks later, CD4 T cells were isolated from the draining lymph nodes and spleens using mouse CD4 MACS microbeads and used immediately for T cell assays.

Flow Cytometric Analysis

B6 primary renal TEC were harvested by light trypsinisation. Before staining with primary antibodies, BM-DC were pre-incubated with anti-mouse CD16/32 mAb for 15 min to block FcR binding. TEC or BM-DC were then incubated with specific primary mAbs for 45 min. Cells were then washed and incubated with the appropriate FITC-conjugated secondary antibody (Ab) for an additional 30 min and analyzed using the FACScan flow cytometer and Cell Quest™ software (Becton Dickinson).

OT-1 CD8 T Cell Activation by TEC or BM-DC

Untreated B6 primary renal TEC, TEC treated with different cytokines (fig. 3 and 4), or LPS matured B6 BM-DC, were pulsed with 10 ng/ml of H-2K^b-restricted $OVA_{257–264}$ peptide or the control peptide β-$Gal_{497–504}$ for 1 h at 37°C. After washing, APC were seeded into 96-well cell culture plates (2×10^4/well) with freshly isolated resting OT-1 CD8 T cells (2×10^5/well) in DMEM medium supplemented with 5% FBS and co-incubated at 37°C to study $OVA_{257–264}$ peptide-specific OT-1 CD8 T cell activation. In some experiments, anti-mouse PD-L1 mAb (10 μg/ml) was added to TEC 1 h prior to the addition of OT-1 CD8 T cells as indicated. The same concentration of isotype mAb was used as control. After 24 h, supernatants from co-cultures were collected to analyze IFN-γ content, using a mouse IFN-γ-specific ELISA kit (OptEIA™ mouse IFN-γ kit, BD Pharmingen).

OT-1 CD8 T Cell Proliferation Assay

To study OT-1 CD8 T cell proliferation induced by renal TEC and BM-DC, untreated B6 TEC, TEC treated with different cytokines, or LPS matured B6 BM-DC were pulsed with 10 ng/ml of $OVA_{257–264}$ peptide or the control peptide β-$Gal_{497–504}$ for 1 h at

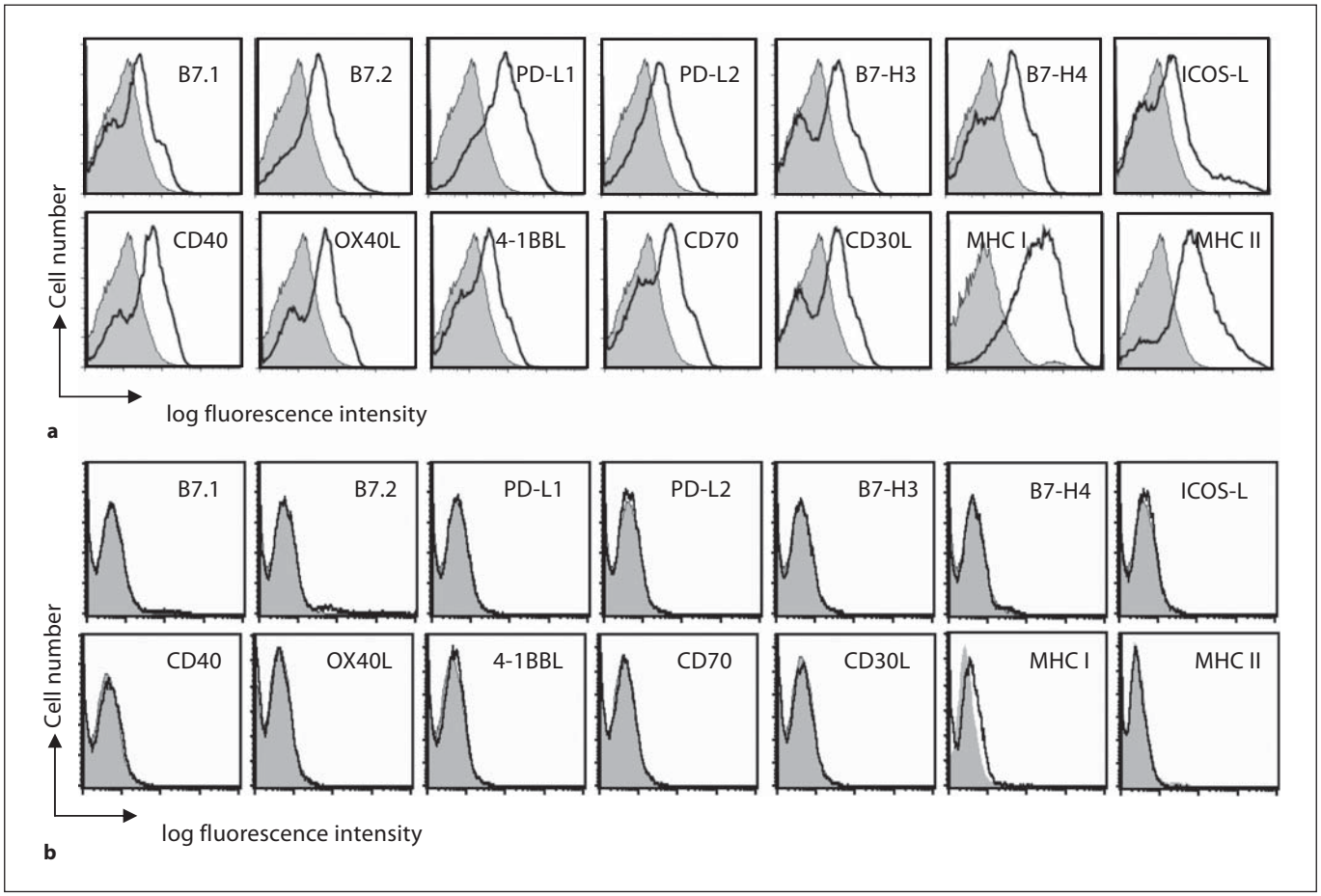

Fig. 1. Constitutive surface expression of costimulatory molecules on BM-DC and on renal TEC. LPS matured murine BM-DC (**a**) or primary renal TEC (**b**) generated from B6 mice were stained with the indicated anti-mouse costimulatory molecule mAbs for flow cytometric analysis.

37°C. After washing, APC were seeded into 96-well plates (2×10^4/well) with freshly isolated resting OT-1 CD8 T cells (2×10^5/well) and incubated at 37°C. T cell proliferation was determined after 96 h by using a non-radioactive colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit according to the manufacturer's protocol (Promega). The proliferation index was calculated as:

$$\text{Proliferation Index} = (OD_{experimental} - OD_{medium})/(OD_{T\ cells\ only} - OD_{medium})$$

Determination of Antigen-Specific CD4 T Cell Proliferation and Cytokine Production Profile

To study the MHC class II-restricted antigen presentation, B6 renal primary TEC were first pretreated with IFN-γ to enhance MHC class II surface upregulation for 48 h. BM-DC were harvested after LPS maturation. Cells were then collected and washed and seeded into 96-well culture plates (2×10^4 APC/well) as described above. The in vivo OVA-primed CD4 T cells (2×10^5/well) were added to the APC in DMEM medium supplemented with 5% FBS and co-incubated at 37°C in the presence of OVA protein (50 μg/ml). At time points 24, 48 and 96 h, supernatants were collected for measuring IL-2, IFN-γ and IL-4 contents, using mouse specific ELISA kits (OptEIA™ mouse ELISA kits, BD Biosciences), according to the manufacturer's protocols. Antigen-specific CD4 T cell proliferation was determined after 96 h as describe above.

Cytotoxicity by Activated OT-1 CD8 CTL to B6 TEC

To measure antigen-specific cytolysis of renal TEC by CTL, activated OT-1 CD8 CTL were added to the cytokine-pretreated, $OVA_{257-264}$ peptide-loaded B6 TEC in 96-well U bottom culture plates at effector/target (E/T) ratios 10 to 1 (2×10^5 CTL and 2×10^4 targets per well) in the presence or absence of anti-mouse PD-L1 mAb (10 μg/ml). After 4 h, 50 μl of supernatants were collected from each well for measuring the amount of lactate dehydrogenase (LDH) released upon cell lysis with the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega), according to the manufacturer's protocol.

Statistics

All experiments were performed in triplicates or quadruplicates and were carried out at least 3 times. Data are expressed as mean ± SD. Statistical analysis was performed using unpaired Student's t test. Significance was accepted at $p \leq 0.05$.

Results

Costimulatory Molecule Expression Profile of Renal TEC and BM-DC

We compared the constitutive surface expression of costimulatory molecules between renal TEC and dendritic cells by flow cytometric analysis. As shown in figure 1, unlike the LPS matured BM-DC generated from B6 mice which expressed most costimulatory or coinhibitory molecules tested on their surface (fig. 1a), B6 renal primary TEC did not constitutively express any tested molecules under non-inflammatory conditions (fig. 1b). Stimulation of B6 TEC with IFN-γ (100 U/ml) for 48 h enhanced the expression of some costimulatory molecules, including PD-L1, ICOS-L, CD40, as well as MHC class I and class II molecules (fig. 2), whereas the surface upregulation of other molecules was not detected with flow cytometry after IFN-γ treatment (fig. 2).

Since PD-L1 was the most prominent molecule upon IFN-γ stimulation, we then examined whether pro- or anti-inflammatory cytokines other than IFN-γ would also enhance the costimulatory molecule expression on TEC, especially PD-L1. For this purpose, B6 TEC were treated with IFN-α, IFN-β, IFN-γ, TNF-α, IL-1, IL-2, IL-4, IL-6, IL-10 or IL-12, alone or in combination, respectively. Figure 3 shows that surface PD-L1 was upregulated by stimulation of TEC with IFN-β and IFN-γ, but not the other tested cytokines under our experimental conditions. Treated TEC with higher concentrations of IFN-β or IFN-γ did not further increase the surface expression of PD-L1 as shown previously [12] and had no effect on altering other costimulatory molecule expression on renal TEC (data not shown). The cytokine combinations of IFN-γ/TNF-α, IFN-α/IFN-β, or TNF-α/IL-1/IL-6 did not show any synergetic effect on PD-L1 upregulation (data not shown). These results demonstrated that the upregulation of costimulatory molecules (e.g. PD-L1, ICOS-L and CD40) on cell surface was mostly limited to the interferons.

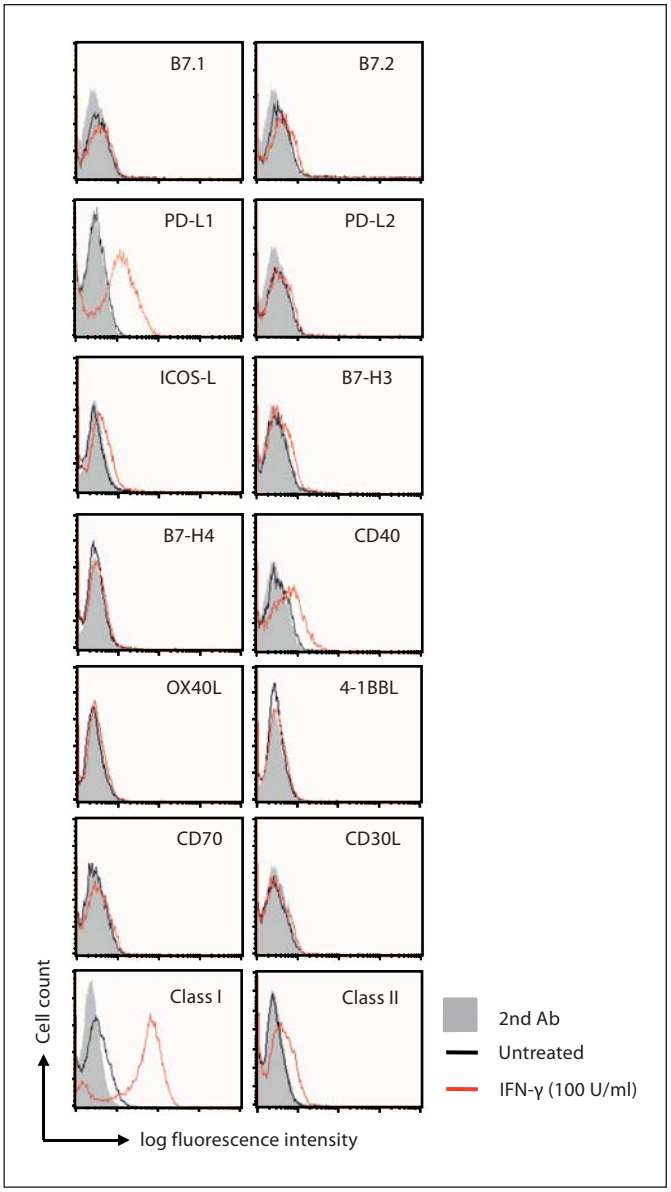

Fig. 2. Costimulatory molecule expression on primary renal TEC stimulated with IFN-γ. B6 primary TEC prestimulated with IFN-γ (100 U/ml) for 48 h were stained with the indicated anti-mouse costimulatory molecule mAbs for flow cytometric analysis. Filled gray histograms represent secondary antibody control stainings.

Activation of $OVA_{257–264}$ Peptide-Specific OT-1 CD8 T Cells by Renal TEC or by BM-DC

To examine the capacity of MHC class I and class II antigen presentation by renal TEC under inflammatory conditions, and to test whether the lack of costimulatory signals between renal TEC/T cells would influence the T cell activation, chicken OVA-specific CD4 and CD8 T cell activation was then investigated, using primary renal TEC or BM-DC as APC, respectively.

To study the $OVA_{257–264}$ peptide-specific CD8 OT-1 T cell activation, B6 renal primary TEC pretreated with different cytokines, or without treatment, were pulsed with

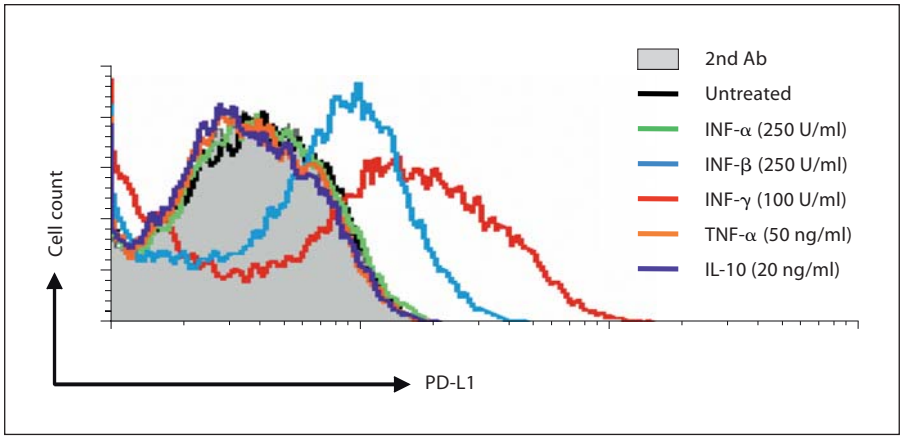

Fig. 3. PD-L1 expression on primary renal TEC treated with different cytokines. B6 primary renal TEC pretreated with the indicated cytokines were stained with anti-mouse PD-L1 mAbs for flow cytometric analysis.

$OVA_{257–264}$ peptide and then co-incubated with resting OT-1 CD8 T cells. As shown in figure 4a, stimulation of OT-1 CD8 T cells by peptide-presenting renal TEC resulted in much lower OT-1 CD8 T cell proliferation as compared to that obtained with BM-DC (proliferation index 6.4 vs. 17). The T cell activation was antigen specific, since co-incubation of OT-1 CD8 T cells with both APC that were pulsed with control peptide β-$Gal_{497–504}$ did not raise T cell proliferation nor IFN-γ production (fig. 4a, b). Similarly, a much lower amount of IFN-γ was detected in the co-culture supernatants of renal TEC/OT-1 CD8 T cells compared to those in the co-culture supernatants of BM-DC/OT-1 CD8 T cells (fig. 4b). In addition, blockade of surface PD-L1 on both IFN-β- and IFN-γ-pretreated renal TEC significantly increased IFN-γ production by OT-1 CD8 T cells (fig. 4b). The PD-L1 blockade effect was also observed on BM-DC surface (fig. 4b). Together, these data demonstrate that renal TEC are poor antigen presenting cells for activating specific CD8 T cells and that PD-L1 molecule functions as a negative costimulatory molecule on both APC.

To further examine whether high PD-L1 surface expression on renal TEC would directly influence CTL activity, the cytotoxicity of OT-1 CD8 CTL was tested by using high or low PD-L1-expressing renal TEC as targets (fig. 3). $OVA_{257–264}$ peptide-dose titration experiments demonstrated that loading IFN-β- or IFN-γ-stimulated TEC with high antigen concentration cannot further increase the cytolyses of those TEC by OT-1 CTL , as compared to the TEC with low PD-L1-expression (untreated) (fig. 5). Considering the fact that surface PD-L1 was upregulated only by IFN-β and IFN-γ but not the other tested cytokines, these results suggested that high PD-L1 surface expression protected renal TEC from the lysis by CTL.

Stimulation of OVA Protein-Specific CD4 T Cells by Renal TEC or by BM-DC

Next, we studied the capacity of presenting MHC class II antigens by renal TEC. OVA-specific CD4 T cell proliferation was examined by using IFN-γ-pretreated B6 TEC (to enhance MHC class II molecule expression, fig. 2) or LPS-matured BM-DC. Similarly to the $OVA_{257–264}$-specific CD8 T cell proliferation, OVA-specific CD4 T cell proliferation induced by renal TEC was much lower than that induced by BM-DC (proliferation index 2.9 vs. 22.3, fig. 6a).

The pattern and kinetics of cytokine production in the supernatants of APC/OVA-specific CD4 T cell co-cultures were also determined (fig. 6b, c). Interestingly, renal TEC-stimulated CD4 T cells produced a high amount of IL-2, a low amount of IFN-γ and a basal amount of IL-4 (fig. 6b and data not shown), indicating that renal TEC-stimulated CD4 T cells did not further differentiate to the Th1 or Th2 subsets. In addition, the cytokine production by renal TEC-stimulated CD4 T cells started to decline after 48 h of stimulation, indicating that this activation was suboptimal and short-termed. On the contrary, BM-DC-activated OVA-specific CD4 T cells secreted a low amount of IL-2, a high amount of IFN-γ and a basal amount of IL-4 (fig. 6c and data not shown), showing a clear Th1 phenotype. Moreover, BM-DC-activated CD4 cells continuously produced a high amount of IFN-γ during the whole experimental period (fig. 6c). Together these data indicate that CD4 T cells stimulated by BM-DC were fully activated.

To test the functional role of epithelial PD-L1 on CD4 T cell activation, anti PD-L1 Abs were also used to block PD-L1 on APC surface at the beginning of the antigen presentation experiments described above. PD-L1 block-

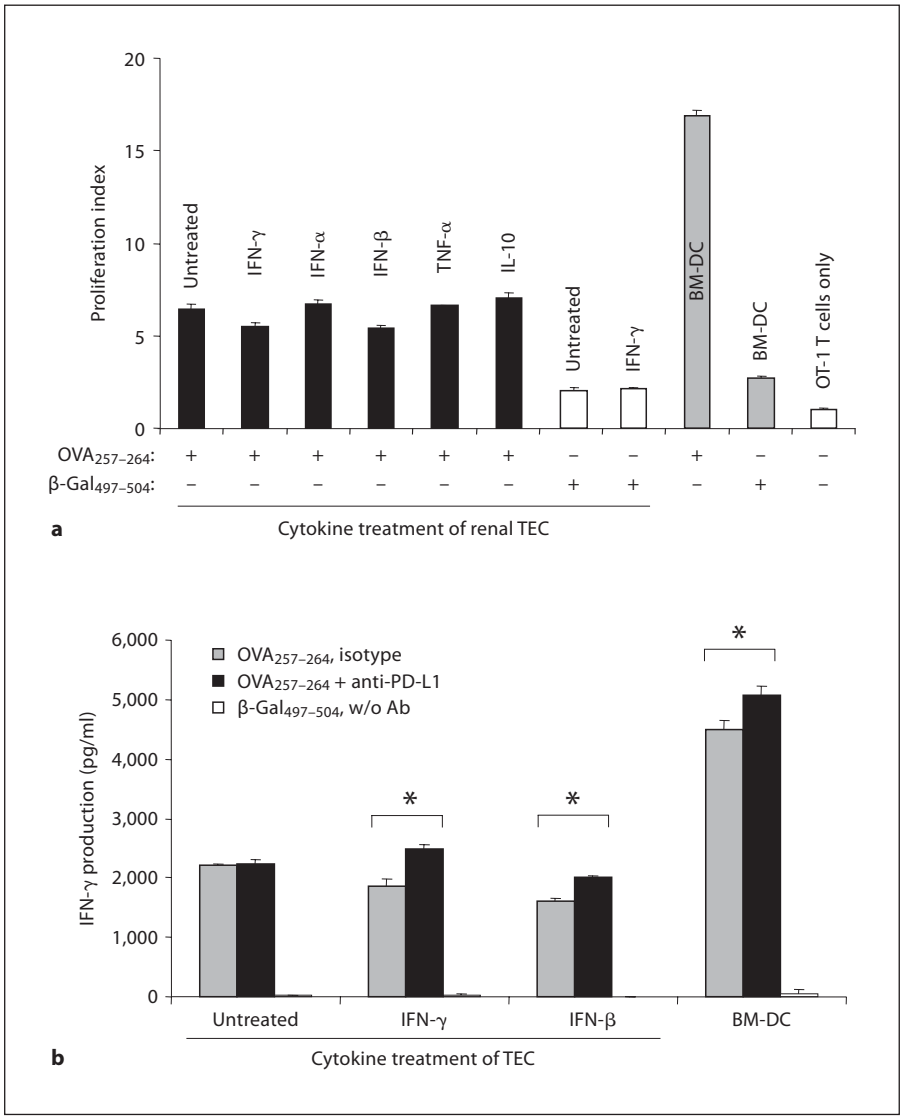

Fig. 4. $OVA_{257–264}$ peptide-specific OT-1 CD8 T cell activation by congeneic renal TEC or by BM-DC. **a** Inflammatory cytokine-pretreated (fig. 3) or untreated B6 primary renal TEC- or LPS-matured BM-DC pulsed with $OVA_{257–264}$ peptide were co-incubated with resting OT-1 CD8 T cells for 96 h for determining the T cell proliferation as described in 'Materials and Methods'. Data represent the mean proliferation index ± SD. **b** Untreated, IFN-β- (250 U/ml) or IFN-γ- (100 U/ml) pretreated and $OVA_{257–264}$ peptide- or control peptide-pulsed renal B6 TEC or BM-DC were co-incubated with resting OT-1 CD8 T cells as described in (**a**), respectively, in the presence or absence of the PD-L1-blocking mAbs (final concentration 10 μg/ml). After 24 h, supernatants were collected to determine the IFN-γ production by IFN-γ ELISA. Data represent the mean amount of IFN-γ ± SD. * $p \leq 0.05$ when compared with TEC without Ab blockade.

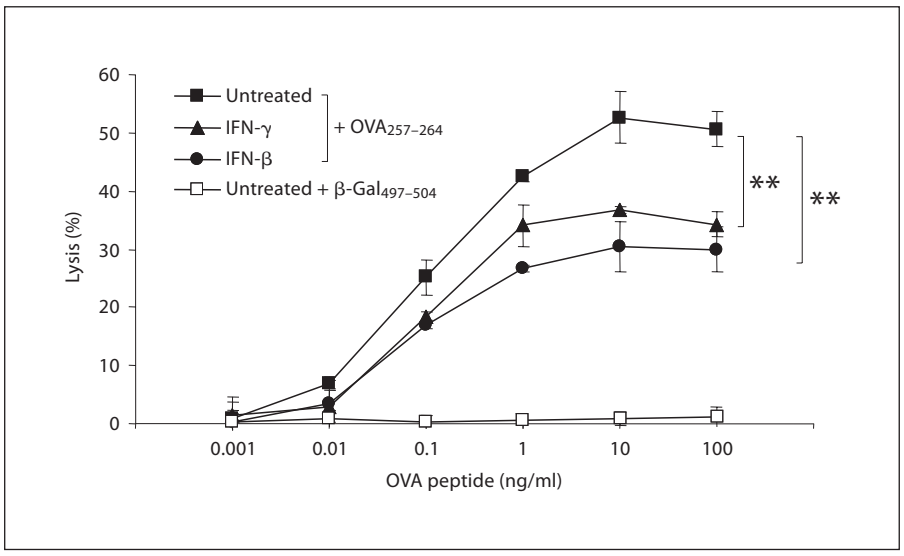

Fig. 5. High PD-L1-expressing renal TEC were more resistant to the cytolysis by activated OT-1 CD8 CTL. IFN-β- (250 U/ml) or IFN-γ- (100 U/ml) pretreated, or untreated B6 TEC pulsed with different concentrations of $OVA_{257–264}$ peptide or control β-$Gal_{497–504}$ peptide were co-incubated with OT-1 CTL to determine their cytolyses as described in 'Materials and Methods'. Data represent the mean % lysis ± SD. ** $p \leq 0.001$, when compared with untreated TEC.

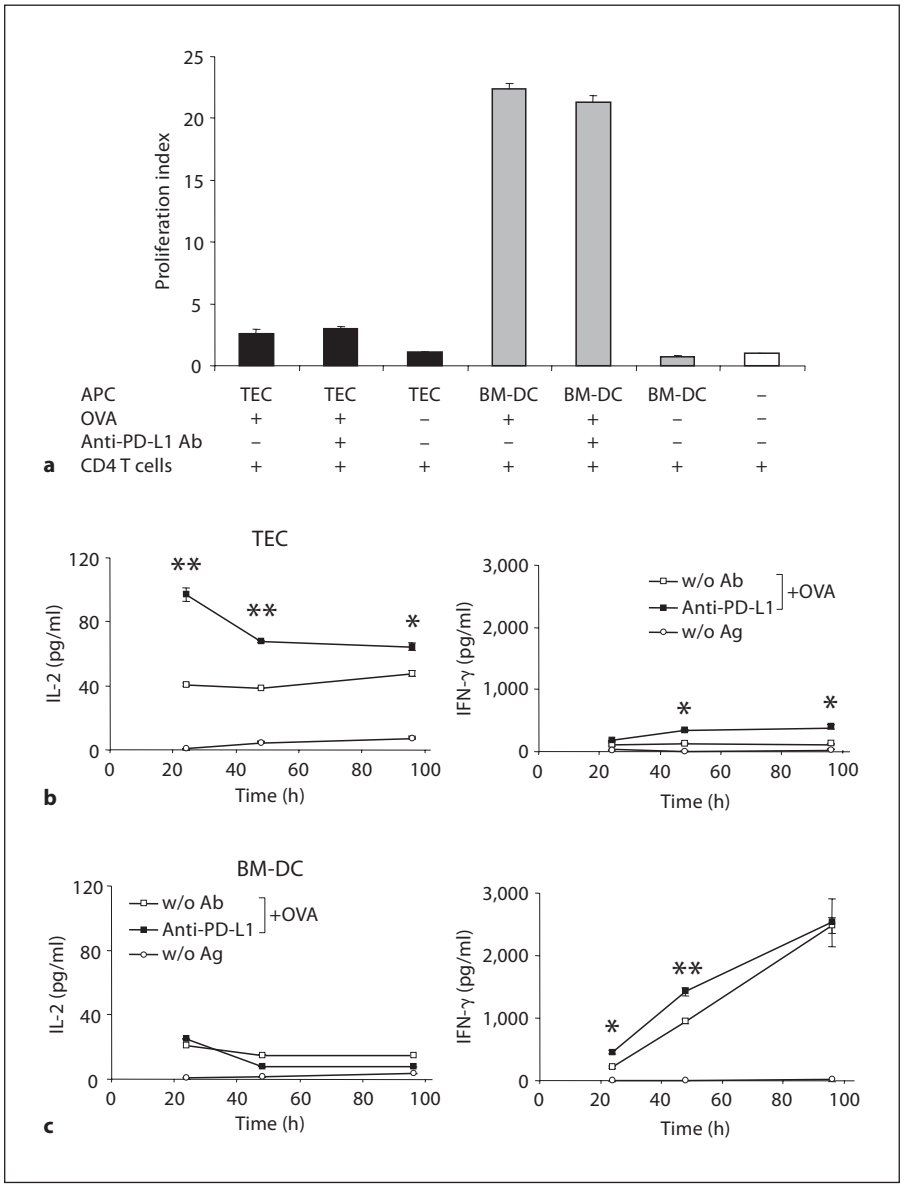

Fig. 6. Comparison of OVA protein-specific CD4 T cell responses induced by autologous renal TEC or by BM-DC. **a** IFN-γ-pretreated, untreated B6 TEC or BM-DC were loaded with OVA protein and incubated with in vitro primed CD4 T cells for 96 h to determine the specific T cell proliferation as described in 'Materials and Methods'. **b** The supernatants of CD4 T cell/APC co-cultures described in (**a**) were collected at time points 24, 48 and 96 h, respectively, to determine the cytokine production profile with specific IL-2 or IFN-γ ELISA. While indicated, anti-PD-L1 Abs were included in the experiments. Data represent the mean amount of cytokine ± SD. * $p \leq 0.05$, ** $p \leq 0.001$, when compared with anti PD-L1 Ab blockade.

ade did not significantly change T cell proliferative responses induced either by renal TEC or by BM-DC (fig. 6a), whereas blockade of PD-L1 on TEC surface significantly elevated IL-2 (2-fold) and IFN-γ (2.5-fold) productions in the supernatants of renal TEC/CD4 T cell co-cultures measured after 24 or 48 h, respectively (fig. 6b). The significant increase of IFN-γ production in BM-DC activated CD4 T cells was also observed after 24 and 48 h (1.5-fold; fig. 6c). The non-effectiveness of PD-L1 Ab blockade in T cell proliferation assays may be due to the dissociation of the Ab/PD-L1 binding or the degradation of the antibodies during 96 h incubation, since the difference between cytokine production in the cell co-cultures with or without Ab blockade also started to diminish after 48 h (fig. 6b, c).

Discussion

In this study, we investigated the profile of costimulatory molecules expressed on renal TEC upon stimulation with various inflammatory cytokines. Compared to dendritic cells, renal TEC were poor antigen-presenting cells for activating both CD4 and CD8 T cells. In an early

study, Singer et al. [16] reported that presentation of antigens by renal TEC to a CD4 T cell clone resulted in functional inactivation of the T cells. Frasca et al. [17] compared the antigen presentation capacity between human primary TEC and EBV transfected B cells for activating CD4 T cells and demonstrated that IFN-γ-treated human primary TEC induced allospecific tolerance. However, the molecular mechanisms involved in the T cell activation by renal TEC are still not fully understood.

The expression profile of MHC and costimulatory molecules on professional or non-professional APC (e.g. renal TEC) can greatly influence the magnitude of T cell activation. BM-DC constitutively expresses a higher quantity of MHC molecules than renal TEC under noninflammatory conditions. However, the handicapped T cell activation induced by renal TEC cannot be simply explained by the MHC molecule expression, since stimulation of renal TEC with pro-inflammatory cytokines is known to effectively upregulate MHC class I and II molecules on TEC, whereas their ability of activating T cells is not significantly improved. We demonstrated in this study that TEC expressed a low level of surface costimulatory molecules under noninflammatory conditions and that only a few costimulatory molecules were upregulated upon stimulation with IFNs. Alternatively, other pathways might also be involved in T cell response and function under inflammatory conditions. Lang et al. reported that, in addition to the upregulation of MHC molecules by LCMV infection, Toll-like receptor (TLR) engagement converted T cell autoreactivity into overt autoimmune disease in a mouse model [18]. Nevertheless, the lack of costimulatory signals on renal TEC might be at least partly responsible for the impaired T cell activation.

The classical costimulatory molecules B7.1 and B7.2 are not expressed by renal TEC, neither at the mRNA level nor at the cell surface [10, 19, 20]. Thus, both B7/CD28 costimulatory and B7/CTLA-4 co-inhibitory pathways cannot play a major role in renal TEC and T cell interaction. Although mRNA transcripts for B7-H3, B7-H4, OX40-L and 4-1BBL were detected in murine primary renal TEC (data not shown), they were not expressed on the surface of renal TEC, even after stimulation with inflammatory cytokines. PD-L1 was the most prominently expressed costimulatory molecule among the IFN-upregulated molecules. Therefore, PD-L1 may provide an alternative negative signaling to control inflammatory responses in the absence of B7/CTLA-4 interaction.

PD-L1 is widely involved in immunoregulatory processes, e.g. tumor escape mechanisms, inflammatory responses, organ-specific autoimmunity, transplantation [4, 21–24]. Thompson et al. [25] recently reported that the upregulation of PD-L1 was associated with a higher mortality in renal cell carcinoma (RCC) patients, suggesting that PD-L1 expressed on tumor cells could directly inhibit functions of infiltrating tumor-specific T cells. Mice which are deficient in PD-L1 and/or PD-1 have a strong tendency to develop autoimmune diseases [26, 27]. Upregulation of PD-L1 was observed in the rejected allografts of kidney, heart, skin and islets of pancreas [8, 28–30], suggesting that PD-L1 plays an important role in the regulation of the rejection process.

We and others previously described that PD-L1 is strongly upregulated by TEC in the rejected murine kidney transplants as well as in inflamed human kidneys [8, 10, 11]. Recently, we demonstrated that renal TEC with high surface PD-L1 induced by IFNs were more resistant to the cytolysis by OT-1 CTL. This lower cytolysis of TEC can be overcome by PD-L1 blockade [12]. In the present study, the blockade of PD-L1 on renal TEC not only increased CD8 T cell activation, but also OVA-specific CD4 T cell proliferation and cytokine production. De Haij et al. [10] observed that human TEC downregulated the CD3/CD28-activated T cell proliferation and modulated the cytokine production profile of T cells. Taken together, we speculate that renal TEC may use PD-L1 to suppress the activity of infiltrating T cells during inflammation, hence, to protect renal tissue from the damage induced by immunopathologic responses. Further in vivo studies are required to explore the protective role of PD-L1 in parenchymal immune responses in the kidney.

Taken together, our data demonstrated that the lack of costimulatory signals between renal TEC and T cells greatly impaired the antigen-specific T cell activation. The limited costimulatory molecule expression on renal TEC may be essential to keep the local immune health in the renal tissues. The IFN-induced PD-L1 upregulation on renal TEC may be crucial to retain the integrity of the kidney under immunopathologic conditions and, therefore, may play an important role in renal parenchymal immunoregulation.

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References

1 Rubin-Kelley VE, Jevnikar AM: Antigen presentation by renal tubular epithelial cells. J Am Soc Nephrol 1991;2:13–26.
2 Meyers CM: New insights into the pathogenesis of interstitial nephritis. Curr Opin Nephrol Hypertens 1999;8:287–292.
3 van Kooten C, Daha MR, van Es LA: Tubular epithelial cells: A critical cell type in the regulation of renal inflammatory processes. Exp Nephrol 1999;7:429–437.
4 Greenwald RJ, Freeman GJ, Sharpe AH: The B7 family revisited. Annu Rev Immunol 2005;23:515–548.
5 Watts TH: TNF/TNFR family members in costimulation of T cell responses. Annu Rev Immunol 2005;23:23–68.
6 Chen L: Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004;4:336–347.
7 Keir ME, Sharpe AH: The B7/CD28 costimulatory family in autoimmunity. Immunol Rev 2005;204:128–143.
8 Schoop R, Wahl P, Le Hir M, Heemann U, Wang M, Wuthrich RP: Suppressed T-cell activation by IFN-gamma-induced expression of PD-L1 on renal tubular epithelial cells. Nephrol Dial Transplant 2004;19:2713–2720.
9 Wahl P, Wuthrich RP: Role of the B7RP-1/ICOS pathway in renal tubular epithelial antigen presentation to CD4 Th1 and Th2 cells. Nephron Exp Nephrol 2004;98:e31–e38.
10 de Haij S, Woltman AM, Trouw LA, Bakker AC, Kamerling SW, van der Kooij SW, Chen L, Kroczek RA, Daha MR, van Kooten C: Renal tubular epithelial cells modulate T-cell responses via ICOS-L and B7-H1. Kidney Int 2005;68:2091–2102.
11 Chen Y, Zhang J, Li J, Zou L, Zhao T, Tang Y, Wu Y: Expression of B7-H1 in inflammatory renal tubular epithelial cells. Nephron Exp Nephrol 2006;102:e81–e92.
12 Waeckerle-Men Y, Starke A, Wuthrich RP: PD-L1 partially protects renal tubular epithelial cells from the attack of CD8 cytotoxic T cells. Nephrol Dial Transplant 2007;22:1527–1536.
13 Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR: T cell receptor antagonist peptides induce positive selection. Cell 1994;76:17–27.
14 Wuthrich RP, Glimcher LH, Yui MA, Jevnikar AM, Dumas SE, Kelley VE: MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. Kidney Int 1990;37:783–792.
15 Waeckerle-Men Y, Gander B, Groettrup M: Delivery of tumor antigens to dendritic cells using biodegradable microspheres. Methods Mol Med 2005;109:35–46.
16 Singer GG, Yokoyama H, Bloom RD, Jevnikar AM, Nabavi N, Kelley VR: Stimulated renal tubular epithelial cells induce anergy in CD4 T cells. Kidney Int 1993;44:1030–1035.
17 Frasca L, Marelli-Berg F, Imami N, Potolicchio I, Carmichael P, Lombardi G, Lechler R: Interferon-gamma-treated renal tubular epithelial cells induce allospecific tolerance. Kidney Int 1998;53:679–689.
18 Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, Odermatt B, Conrad C, Ittner LM, Bauer S, Luther SA, Uematsu S, Akira S, Hengartner H, Zinkernagel RM: Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 2005;11:138–145.
19 Hagerty DT, Evavold BD, Allen PM: Regulation of the costimulator B7, not class II major histocompatibility complex, restricts the ability of murine kidney tubule cells to stimulate CD4 T cells. J Clin Invest 1994;93:1208–1215.
20 Wahl P, Schoop R, Bilic G, Neuweiler J, Le Hir M, Yoshinaga SK, Wuthrich RP: Renal tubular epithelial expression of the costimulatory molecule B7RP-1 (inducible costimulator ligand). J Am Soc Nephrol 2002;13:1517–1526.
21 Blank C, Gajewski TF, Mackensen A: Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother 2005;54:307–314.
22 Rini BI: Current status and future directions of molecular markers in renal cell carcinoma. Curr Opin Urol 2006;16:332–336.
23 Dong H, Chen X: Immunoregulatory role of B7-H1 in chronicity of inflammatory responses. Cell Mol Immunol 2006;3:179–187.
24 Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH: Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. J Exp Med 2006;203:2223–2227.
25 Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, Sengupta S, Frank I, Parker AS, Zincke H, Blute ML, Sebo TJ, Cheville JC, Kwon ED: Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 2006;66:3381–3385.
26 Nishimura H, Nose M, Hiai H, Minato N, Honjo T: Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;11:141–151.
27 Latchman YE, Liang SC, Wu Y, Chernova T, Sobel RA, Klemm M, Kuchroo VK, Freeman GJ, Sharpe AH: PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. Proc Natl Acad Sci USA 2004;101:10691–10696.
28 Koga N, Suzuki J, Kosuge H, Haraguchi G, Onai Y, Futamatsu H, Maejima Y, Gotoh R, Saiki H, Tsushima F, Azuma M, Isobe M: Blockade of the interaction between PD-1 and PD-L1 accelerates graft arterial disease in cardiac allografts. Arterioscler Thromb Vasc Biol 2004;24:2057–2062.
29 Sandner SE, Clarkson MR, Salama AD, Sanchez-Fueyo A, Domenig C, Habicht A, Najafian N, Yagita H, Azuma M, Turka LA, Sayegh MH: Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. J Immunol 2005;174:3408–3415.
30 Gao W, Demirci G, Strom TB, Li XC: Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. Transplantation 2003;76:994–999.

CHAPTER 2

PD-L1 partially protects renal tubular epithelial cells from the attack of $CD8^+$ cytotoxic T cells

Ying Waeckerle-Men, **Astrid Starke** and Rudolf P. Wüthrich

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PD-L1 partially protects renal tubular epithelial cells from the attack of CD8$^+$cytotoxic T cells

Ying Waeckerle-Men[1], Astrid Starke[1] and Rudolf P. Wüthrich[1,2]

[1]Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zürich-Irchel and [2]Clinic for Nephrology, University Hospital Zürich, Switzerland

Abstract

Background. Activated infiltrating T cells play a crucial role in nephritic inflammation via the direct interaction with proximal tubular epithelial cells (TEC). Under inflammatory conditions, major histocompatibility complex class I and II molecules are upregulated on the surface of renal TEC, enabling them to function as 'non-professional' antigen-presenting cells (APC) to activate T cells, and, in turn to be targeted by cytotoxic T lymphocytes (CTL) to cause tissue damage. It is known that co-stimulatory (e.g. B7/CD28) and co-inhibitory (e.g. PD-L1/PD-1) signals regulate and determine the magnitude of T cell responses. In this study, we examined the expression of co-stimulatory molecule PD-L1 by renal TEC and the functional role of renal PD-L1/PD-1 pathway in regulating CD8$^+$ T cell responses induced by antigen-presenting renal TEC.

Methods. Renal TEC were treated with type I and type II interferons (IFN-α, IFN-β or IFN-γ). PD-L1 expression was then determined with flow cytometry and RT-PCR. To investigate the functional role of renal epithelial PD-L1 on CD8$^+$ CTL responses, H-2K^b-restricted, $OVA_{257–264}$ peptide-specific CD8$^+$ T cells isolated from OT-1 T cell receptor transgenic mice were co-incubated with IFN-stimulated, $OVA_{257–264}$ peptide-pulsed congeneic TEC. The activation of OT-1 CD8$^+$ CTL was estimated either by IFN-γ production in the supernatants of co-cultures or by CTL activity.

Results. TECs do not constitutively express PD-L1 on their surface. However, a strong and dose-dependent upregulation of PD-L1 was observed on TEC after stimulation with IFN-β or IFN-γ, but not with IFN-α. $OVA_{257–264}$ peptide pulsed-TEC were able to activate OT-1 CD8$^+$ T cells, indicated by the high amount of IFN-γ production and cytolysis of TEC. Blockade of epithelial PD-L1 with specific mAb significantly increased OT-1 CD8$^+$ T cell activity, indicating that the PD-L1 pathway has a negative effect on CD8$^+$ T cell responses. Moreover, IFN- β- or IFN-γ-stimulated TEC with high surface PD-L1 expression were more resistant to the cytolysis by OT-1 CTL.

Conclusion. Together our data reveal that the renal PD-L1/PD-1 pathway has a negative effect on CD8$^+$ CTL activation. PD-L1 might, therefore, act as a protective molecule on TEC, downregulating the cytotoxic renal parenchymal immune response.

Keywords: CD8$^+$ T cells; interferon; PD-L1; renal tubular epithelial cells

Introduction

The proximal renal tubular epithelium is an important target in tubulointerstitial immune-mediated kidney diseases [1]. Activated infiltrating T cells play a crucial role in nephritic inflammation via the direct interaction with renal proximal tubular epithelial cells (TEC). Thus, infiltrating CD4$^+$ and CD8$^+$ T cells surrounding the tubular epithelium are found in abundance in most tubulointerstitial disease processes, including tubulointerstitial nephritis and renal allograft rejection [2,3]. The ability of renal TEC to promote T cell activation is attributable to their expression of major histocompatibility complex (MHC) class I and class II molecules and co-stimulatory molecules. Renal TECs act as non-professional antigen-presenting cells (APC) to trigger specific T cell responses, and can in turn be targeted by antigen-specific cytotoxic T cells (CTL) during immune-mediated kidney diseases. Although it is well known that CD8$^+$ T cells are the predominant effector cells in tubulointerstitial renal injury and allogeneic kidney transplantation, the regulatory mechanisms of CD8$^+$ T cell responses to TEC have not been fully elucidated yet [4–8]. In particular, studies examining the role played by B7-related

Correspondence and offprint requests to: Dr Y. Waeckerle-Men, Institute of Physiology, University of Zurich-Irchel, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.
Email: ying.waeckerle-men@access.unizh.ch

co-stimulatory molecules on $CD8^+$ T cell responses to TEC are lacking.

Co-stimulatory pathways effectively regulate T cell activation and tolerance [9,10]. The classical co-stimulatory molecules expressed on professional APC include B7.1, B7.2 and CD40. Interactions of B7/CD28 and CD40/CD40L provide positive signals to upregulate T cell responses, whereas the B7/CTLA-4 pathway leads to suppression of T cell responses. Recently, several novel co-stimulatory and co-inhibitory molecules have been discovered. A negative T cell regulatory pathway which could potentially be important for immune-mediated tubulointerstitial injury is represented by the interaction between programmed death-1 (PD-1) expressed on activated T cells, and its two ligands PD-L1 and PD-L2 which are expressed on APC [9–11]. Recently, we and others have described that PD-L1 is strongly upregulated by TEC in rejected kidney transplants as well as in inflamed kidneys [2,3,12], suggesting that it may play an important regulatory role in immune-mediated renal injury.

We and others have previously shown that IFN-γ treatment rapidly and strongly upregulated PD-L1 but not PD-L2 on cultured murine TEC, and that blockade with mAbs of PD-L1 on TEC significantly increased antigen-specific $CD4^+$ T cell responses [2]. Thus far the functional role of the renal epithelial PD-L1/PD-1 pathway in $CD8^+$ CTL responses has not been investigated. In this study we first examined in detail the differential effect of type I and type II interferons (IFNs) on PD-L1 and MHC class I expression in renal TEC, then examined the functional role of the PD-L1/PD-1 pathway on the antigen-specific activation of $CD8^+$ T cells by TEC, and the cytolytic effect to TEC by activated $CD8^+$ CTL.

Materials and methods

General reagents

Cell culture reagents were obtained from Invitrogen (Gaithersburg, MD) and Sigma (St. Louis, MO). Recombinant mouse IFN-α, IFN-β and IFN-γ were purchased from R&D Systems (Oxford, UK). Anti-mouse PD-L1, PD-1 and MHC class I monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA). Biotin-conjugated anti-mouse $H\text{-}2K^k$ was obtained from BD Biosciences (San Jose, CA). Chicken ovalbumin peptide 257-264 ($OVA_{257–264}$, amino acid sequence SIINFEKL) was purchased from Proimmune (London, UK). A control $H\text{-}2K^b$ restricted β-galactosidase peptide 497–504 ($\beta\text{-}Gal_{497–504}$, amino acid sequence ICPMYARV) was a kind gift from Prof. Groettrup of Constance University, Germany. Anti-mouse CD8a (Ly-2) MACS microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Animals, cell lines and renal proximal TEC cultures

T cell receptor (TCR) transgenic OT-1 mice [13] were obtained from Jackson Laboratories (Bar Harbor, ME). C57BL/6 (B6) and C3H/HeN (C3H) mice were purchased from Harlan (Horst, Netherlands). All animals were used at 8–16 weeks of age. The study protocol was approved by the regulatory commission for animal studies of the Canton Zurich, Switzerland. Primary cultures of murine renal proximal TEC were prepared as described previously [14]. Primary renal TEC and SV40-transformed murine renal proximal TEC line C1.1 were cultured on collagen-coated cell culture plates in modified K1 medium as previously described [14]. A murine macrophage cell line RAW 264.7 and EL-4 cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) with Glutamax®, supplemented with 1000 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS. All cells were maintained at 37°C with 5% CO_2.

RNA extraction and RT-PCR analysis

Total RNA from cell cultures was extracted using the RNeasy® mini kit (Qiagen, Valencia, CA). All samples were quantified by the measurement of the optical density at 260 nm, and equal amounts were amplified by reverse transcription-polymerase chain reaction (RT-PCR) (Qiagen® OneStep PCR kit, Qiagen). Primer sequences of PD-L1 were determined as previously described [2]. To ensure even amounts of template, the rat housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was co-amplified as described previously [15]. Reaction mixtures were separated on 1.5% agarose gels containing ethidium bromide, and bands were detected under UV light and analysed with the Bio-Image System Chemidoc™ XRS (Bio-Rad, Hercules, CA).

Flow cytometric analysis

RAW264.7, the renal TEC line C1.1 and primary cultures of renal TEC were stimulated with IFN-α, IFN-β or IFN-γ for 48 h. Cells were harvested by light trypsinization, washed twice with Hanks' Balanced Salt Solution (HBSS, Invitrogen) and suspended in PBS containing 2% FBS and 0.1% sodium azide. Before staining with the primary antibody, macrophages were pre-incubated with anti-mouse CD16/CD32 Ab for 15 min to block FcR binding. After incubating with primary mAbs for 45 min on ice, cells were washed twice and incubated with the appropriate FITC-conjugated secondary Ab for 30 min. Cells were then washed and analysed using an FACScan flow cytometer and the Cell Quest™ software (Becton Dickinson).

Isolation of $CD8^+$ T cells from OT-1 mice

Spleen and lymph nodes (LN) were harvested from 8–16-week-old naïve OT-1 mice. $CD8^+$ cells were isolated using anti-mouse CD8a (Ly-2) MACS microbeads according to the manufacturer's protocol. The freshly isolated OT-1 $CD8^+$ T cells were either used immediately for antigen presentation assays, or were activated *in vitro*. To activate OT-1 CTL, OT-1 $CD8^+$ T cells were stimulated with B6 splenocytes pulsed with 0.1 µg/ml of $OVA_{257–264}$ peptide and were incubated in DMEM medium supplemented with 5 µM 2-mercaptomethanol, 1% HEPES, 1000 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS at a T cell to APC ratio of 1:5. After 6 days of stimulation, activated OT-1 CTL were used for CTL assays.

Antigen presentation of renal B6 TEC to resting OT-1 CD8$^+$ T cells

To study the antigen presentation of the H-2K^b-restricted peptide $OVA_{257–264}$ by congeneic primary TEC to OT-1 CD8$^+$ T cells, B6 primary TEC were pre-treated with IFNs for 48 h. Cells were washed to remove excess cytokines and were harvested by light trypsinization. The cells were then pulsed with 0.1 μg/ml of $OVA_{257–264}$ peptide or the control H-2K^b-restricted β-$Gal_{497–504}$ peptide for 1 h at 37°C. After washing, TEC were counted and seeded in 96-well U-bottom cell culture plates (Costar) in DMEM medium supplemented with 5% FBS, 1000 U/ml of penicillin and 100 μg/ml of streptomycin. The resting OT-1 CD8$^+$ T cells were added to TEC cultures and were co-incubated at 37°C. Anti-mouse PD-L1 mAb (10 μg/ml) were added to TEC 1 h prior to the addition of OT-1 CD8$^+$ T cells as indicated. After 24 h, supernatants from co-cultures were collected and analysed for IFN-γ content using a mouse IFN-γ-specific ELISA kit (OptEIA^{TM} mouse IFN-γ kit, BD Pharmingen). In control experiments, $OVA_{257–264}$ or β-$Gal_{497–504}$ peptide-pulsed EL-4 cells were used.

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays (Diaclone, Besançon, France) were also used to determine the IFN-γ production induced by resting OT-1 CD8$^+$ T cells after activation by $OVA_{257–264}$-presenting congeneic TEC. Briefly, untreated B6 TEC or EL-4 cells were pulsed with 0.1 μg/ml of $OVA_{257–264}$ or β-$Gal_{497–504}$ peptide for 1 h at 37°C. The cells were then washed and seeded into the ELISPOT plate. Resting OT-1 CD8$^+$ T cells were added to the TEC cultures and were co-incubated for 18 h at 37°C. ELISPOT plates were developed according to the manufacturer's protocol.

Cytotoxicity by activated OT-1 CD8$^+$ CTL to B6 TEC

To measure antigen-specific cytolysis of renal TEC by CTL, activated OT-1 CD8$^+$ CTL were added to IFN-pre-treated, $OVA_{257–264}$ peptide-loaded B6 TEC in 96-U bottom cell culture plates at various effector/target (E/T) ratios, in the presence or absence of anti-mouse PD-L1 mAb (10 μg/ml). After 4 h of incubation, 50 μl of supernatants were collected from each well for measuring the amount of lactate dehydrogenase (LDH) released upon cell lysis by using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega) according to the manufacturer's protocol. The percentage of target lysis was calculated as follows:

$$\%\ \text{lysis} = 100 \times (\text{experimental LDH release} - \text{spontaneous LDH release of targets} - \text{spontaneous LDH release from T cells})/(\text{maximum LDH release} - \text{spontaneous LDH release of targets}).$$

Statistics

Results from IFN-γ ELISA, ELISPOT or CTL assays are expressed as means ± SD of triplicate or quadruplicate determinations from the representative experiments that gave rise to similar results. Statistical analysis was performed using unpaired Student's *t*-test. Significance was accepted at $P \leq 0.05$.

Results

Stimulation of renal proximal TEC with IFN-β and IFN-γ upregulated PD-L1

Murine macrophage RAW246.7 cells and murine primary proximal TEC cultures prepared from C57BL/6J (B6 TEC) mice were stimulated with 200 U/ml of IFN-β or 100 U/ml of IFN-γ for 1–4 h for determining changes in mRNA expression of PD-L1. As shown in Figure 1, PD-L1 mRNA was constitutively detected in macrophages and renal TEC. A strong increase of steady-state PD-L1 mRNA levels was observed in macrophages after 1 and 4 h of stimulation with either IFN-β or IFN-γ (Figure 1A), in renal B6 TEC after 1 and 4 h of stimulation with IFN-β, and slightly delayed after 4 h with IFN-γ (Figure 1B). Stimulation of TEC with IFN-α even at a higher concentration (up to 1000 U/ml) did not increase PD-L1 mRNA expression in TEC (data not shown).

The surface expression of PD-L1 and MHC class I on TEC was examined after stimulation with IFN-β or IFN-γ for 48 h. Expression of PD-L1 on untreated C1.1 or primary C3H renal TEC cultures (the original of C1.1 cells) was negligible, contrasting with RAW 264.7 macrophages which constitutively express surface PD-L1 (Figure 2A). Stimulation with IFN-β or IFN-γ strongly upregulated PD-L1 (Figure 2A) and MHC class I molecules (Figure 2B) on all cells tested. The upregulation of both PD-L1 and MHC class I molecules on IFN-β or IFN-γ-stimulated renal TEC was dose-dependent (our previous results [2] and Figure 3). Although stimulation of TEC with IFN-α did not increase PD-L1 surface expression even when a high dose (1000 U/ml) was used, a dose-dependent, strong increase of MHC class I expression was observed (Figure 3B), suggesting that IFN-α and IFN-β use different pathways to activate

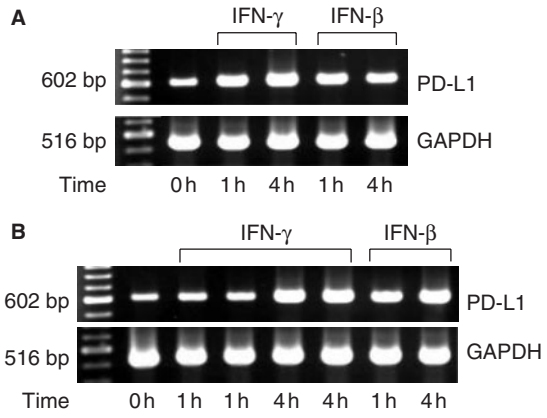

Fig. 1. RT-PCR analysis of PD-L1 mRNA expression in renal TEC treated with IFNs. Murine macrophage RAW264.7 cells (positive control cell line) (**A**) and primary renal TEC cultures generated from B6 mice (**B**) were treated with IFN-β (200 U/ml) or IFN-γ (100 U/ml) for 1 or 4 h. Results are representative of two independent experiments which gave similar results.

antigen-presenting cells. Similar results were also obtained by IFN-stimulation of primary TEC that were generated from different mouse strains, including C57BL/6, C3H, BALB/c and AKR/J (data not shown), indicating that this phenomenon is not limited to a specific cell line or mouse strain.

Activation of resting OT-1 CD8$^+$ T cells by TEC

We used CD8$^+$ T cells isolated from OT-1 TCR transgenic mice to examine the ability of renal TEC to present MHC class I-restricted antigenic peptide. OT-1 mice contain transgenic TCR genes in CD8$^+$ T cells, which are designed to recognize the chicken ovalbumin protein residues 257–264 in the context of H-2K^b ($OVA_{257–264}$) [13]. FACS analysis showed that resting OT-1 CD8$^+$ T cells express PD-L1 but not PD-1, whereas both molecules were strongly upregulated on *in vitro*-activated OT-1 CTL (Figure 4).

Antigen titration experiments were then performed to examine the capacity of presenting $OVA_{257–264}$ peptide to the resting OT-1 CD8$^+$ T cells by renal B6 TEC. Similar amounts of IFN-γ were detected from supernatants of OT-1 CD8$^+$ T cell cultures sensitized either by $OVA_{257–264}$ pulsed congeneic TEC (from C57BL/6 mice) or by EL-4 cells (Figure 5A). These results were further confirmed by IFN-γ ELISPOT assays. As shown in Figure 5B and C, resting OT-1 CD8$^+$ T cells activated by either OVA peptide-presenting B6 TEC or EL-4 cells gave rise to a comparable level of IFN-γ production when captured and visualized by IFN-γ ELISPOTs. These results demonstrate that renal TEC have a strong capacity of presenting MHC class I-restricted antigenic peptides to activate antigen-specific CD8$^+$ T cells *in vitro*.

PD-L1 on renal TEC inhibited antigen presentation to OT-1 CD8$^+$ T cells

We studied next whether PD-L1 was able to regulate CD8$^+$ T cell responses. For this purpose, TEC were first stimulated with IFN-β or IFN-γ for 48 h to

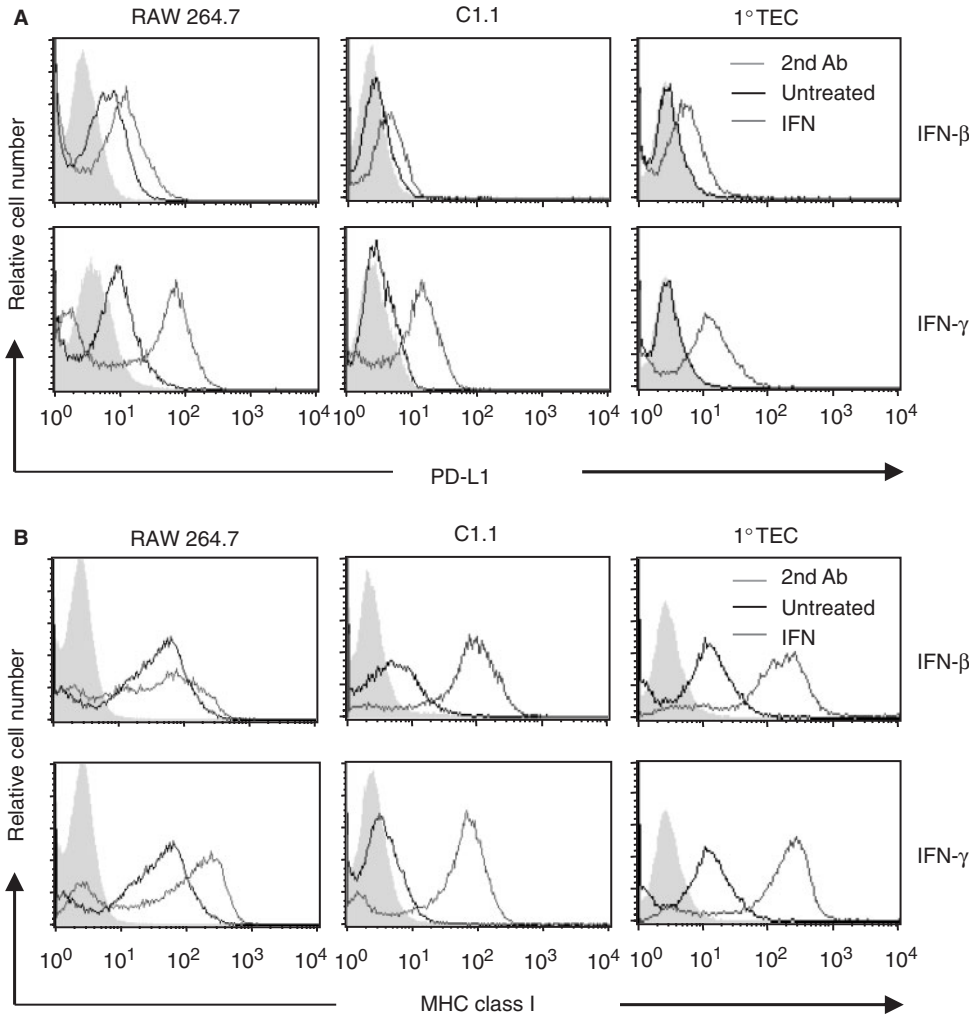

Fig. 2. PD-L1 and MHC class I expression on renal TEC stimulated with IFN-β (200 U/ml) or IFN-γ (100 U/ml) for 48 h. Murine macrophage line RAW264.7, renal TEC line C1.1 and primary TEC cultures generated from C3H mice (1° TEC) were stained with anti-mouse PD-L1 (**A**) or MHC class I (**B**) mAbs for flow cytometric analysis. Results are representative of four independent experiments.

upregulate PD-L1. Cells were then collected, washed and loaded with $OVA_{257–264}$ peptide. Anti-PD-L1 mAb was added to block PD-L1 on TEC before activating the resting OT-1 $CD8^+$ T cells. Blockade of PD-L1 significantly increased OT-1 $CD8^+$ T cell activation, as revealed by higher amounts of IFN-γ, compared to those with TEC without PD-L1 blockade (Figure 6). The reaction was antigen-specific and MHC class I-restricted, since irrelevant β-$Gal_{497–504}$ peptide-pulsed TEC failed to induce OT-1 T cell activation and C1.1 cells (H-$2K^k$) pulsed with $OVA_{257–264}$ peptide did not induce IFN-γ production by OT-1 T cells (data not shown). These results indicated that PD-L1-expressing renal epithelial cells modulated the antigen-specific $CD8^+$ T cell activation.

PD-L1 partially protected TEC from cytolysis by OT-1 CTL

We then examined whether OVA-presenting TEC would be the direct targets of OT-1 $CD8^+$ CTL, and whether epithelial PD-L1 plays a functional role on CTL responses. For this purpose, $OVA_{257–264}$ peptide-pulsed B6 renal TEC, pretreated with IFN-α, IFN-β and IFN-γ or untreated, were used as target cells to study the cytotoxicity of OT-1 $CD8^+$ T cells. Resting OT-1 $CD8^+$ T cells isolated from naïve OT-1 mice failed to kill $OVA_{257–264}$ peptide-presenting target cells, including renal B6 TEC or EL-4 cells (data not shown). In contrast, *in vitro*-stimulated/activated OT-1 $CD8^+$ T cells demonstrated a strong cytotoxicity when $OVA_{257–264}$ peptide-presenting B6 TEC or EL-4 cells were used as targets (Figure 7A). Only basal cytolysis of target cells was observed when TEC and EL-4 cells were pulsed with the irrelevant H-$2K^b$ peptide β-$Gal_{497–504}$ (Figure 7A). Importantly, when IFN-β and IFN-γ-stimulated TEC were used as targets in the same CTL assay, significantly lower cytolysis was observed compared with untreated TEC. The lysis of TEC treated with IFN-α did not show significant difference when compared with untreated TEC (Figure 7A). Considering that IFN-β or IFN-γ treatment upregulated high-surface PD-L1 molecule expression, this may be indirect evidence that the surface PD-L1 protects TEC from the cytolysis by CTL. To further clarify this point, anti-PD-L1 antibodies were added to block the surface PD-L1 during CTL assay. As shown in Figure 7B, the PD-L1 Ab blockade led to a significant increase of cytolysis of TEC treated with IFN-β or IFN-γ, respectively, while adding anti-PD-L1 mAb to untreated or IFN-α-treated TEC did not alter the cytolytic effect of OT-1 $CD8^+$ T cells. Taken together, these results demonstrate that PD-L1 expressed on renal TEC plays an inhibitory role on CTL responses, reducing T cell activation and protecting TEC from the killing by $CD8^+$ CTL.

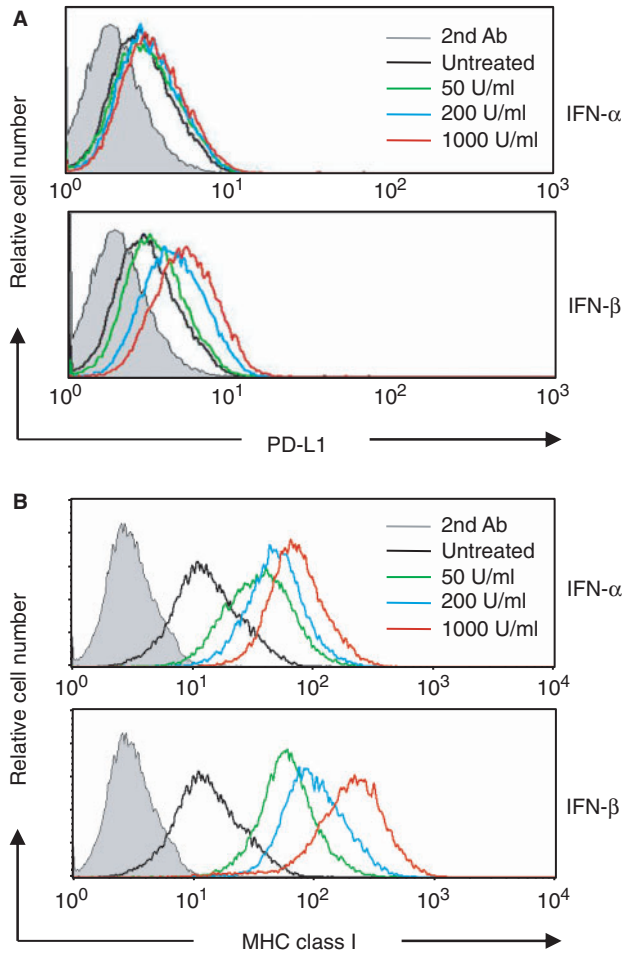

Fig. 3. PD-L1 and MHC class I expression on renal TEC stimulated with type I IFNs. C1.1 cells were stimulated with the indicated concentrations of IFN-α or IFN-β for 48 h, and were stained with anti-mouse PD-L1 (**A**) or MHC class I (**B**) mAbs for flow cytometric analysis. Results are representative of two independent experiments.

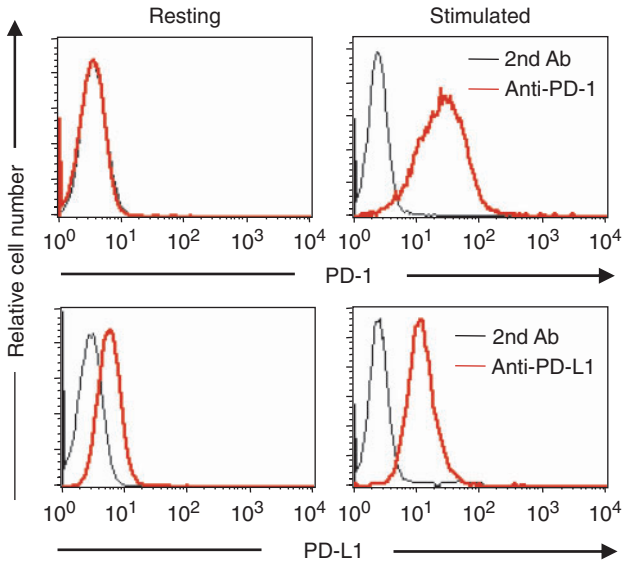

Fig. 4. Flow cytometric analysis of PD-1 and PD-L1 expression on OT-1 $CD8^+$ T cells. Resting or $OVA_{257–264}$ peptide-stimulated OT-1 $CD8^+$ T cells were stained with anti-mouse PD-1 or PD-L1 mAbs for flow cytometric analysis. Results are representative of two independent experiments.

Discussion

In this study we demonstrate that the expression of PD-L1 on TEC is strongly and rapidly upregulated by IFN-β and IFN-γ, but not by IFN-α. Furthermore, we show that the PD-L1/PD-1 pathway inhibits antigen-specific $CD8^+$ T cell activation by renal TEC. In addition, the blockade of PD-L1 on TEC leads to an enhanced cytolytic activity of CTL in an MHC class I-restricted manner. PD-L1 on renal TEC, therefore, provides a negative signal for antigen-specific $CD8^+$ T cell responses.

$CD8^+$ T cells are major contributors in cell-mediated immune responses in tubulointerstitial renal diseases and are responsible for the tubular destruction [4,16]. However, the mechanisms that regulate the intrarenal $CD8^+$ T cell activity have not been investigated in detail. Apart from MHC class I complex/TCR interactions that are crucial for initiating T cell activation, a variety of co-stimulatory and co-inhibitory pathways are known to influence the cytolytic potential of $CD8^+$ T cells [9,10,17]. Among those co-stimulatory pathways, B7/CD28 provides the most prominent activation signal for $CD8^+$ T cells. However, as B7.1 and B7.2 are hardly expressed by renal TEC, the relevant co-stimulatory pathways might be different for TEC-driven $CD8^+$ T cell responses. Similarly, the co-inhibitory B7/CTLA-4 interaction might also not operate on TEC for the same lack of expression of B7 molecules. Considering that only limited co-stimulatory or co-inhibitory molecules such as PD-L1, ICOS-L and CD40 are expressed in renal TEC (our unpublished data), thus PD-L1 may be one of the major co-inhibitory molecules regulating renal epithelial $CD8^+$ CTL responses. Our current findings that the epithelial PD-L1/PD-1 pathway has an immunological regulatory function on $CD8^+$ CTL responses are also consistent with other studies, including cancer immunity, pathogen-induced $CD8^+$ T cell responses, autoimmune diseases and organ transplantation [9,10,18].

PD-L1 is aberrantly expressed by various human carcinomas [18–23]. The expression of PD-L1 by tumour cells can impair tumour-specific

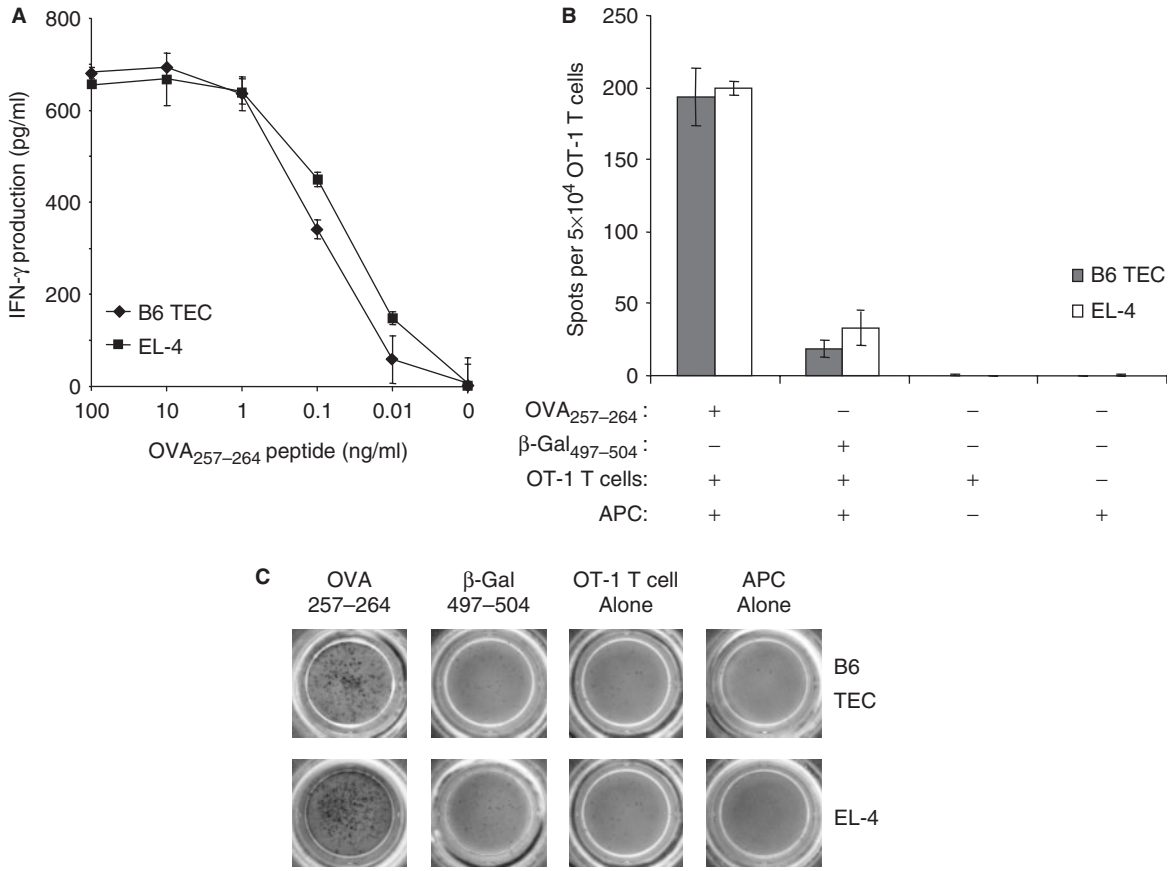

Fig. 5. OVA-presenting renal TEC activate resting OT-1 $CD8^+$ T cells to produce high amounts of IFN-γ. (**A**) Antigen titration assay. Untreated primary B6 TEC or EL-4 cells (1×10^4 per well) pulsed with increasing concentrations of $OVA_{257–264}$ peptide were co-incubated with resting OT-1 $CD8^+$ T cells (5×10^4 per well). After 24 h, OT-1 $CD8^+$ T cell activation was determined with IFN-γ ELISA. Results represent the mean amount of IFN-γ ± SD. (**B** and **C**) IFN-γ ELISPOT assay. Untreated B6 TEC or EL-4 cells (1×10^4 per well) were pulsed with 100 ng/ml of $OVA_{257–264}$ or β-$Gal_{497–504}$ peptide, then were co-incubated with resting OT-1 $CD8^+$ T cells (5×10^4 per well) in pre-coated murine IFN-γ ELISPOT plates. After 18 h, plates were developed, and spots were counted as described in 'Materials and methods'. Wells containing only TEC or OT-1 $CD8^+$ T cells were included as controls. Data represent the mean spots per well ± SD. Representative individual ELISPOT wells with different experimental setting are shown in **C**.

T cell functions, resulting in defective host anti-tumour immunity. For example, high PD-L1 expression in human renal cell carcinomas is correlated with tumour invasiveness and markedly increased the risk of death from cancer [19]. In an animal study, resistance of PD-L1-positive tumour cells to the cytolysis by tumour-specific CTL has been observed, and this could be abrogated by the treatment with anti-PD-L1 or anti-PD-1 antibodies [24].

Interesting results have been obtained regarding the role of PD-L1 in chronic infections. Barber *et al.* [25] demonstrated that the *in vivo* blockade of either PD-L1 or its receptor PD-1 with antibodies enhanced lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cell responses, and restored the function of those 'exhausted' $CD8^+$ T cells during chronic LCMV infection. It is tempting to speculate that the IFN-stimulated expression of PD-L1 on TEC could protect intratubular viruses such as Epstein–Barr or the BK polyoma virus, promoting, therefore, their cytopathic effects [26,27].

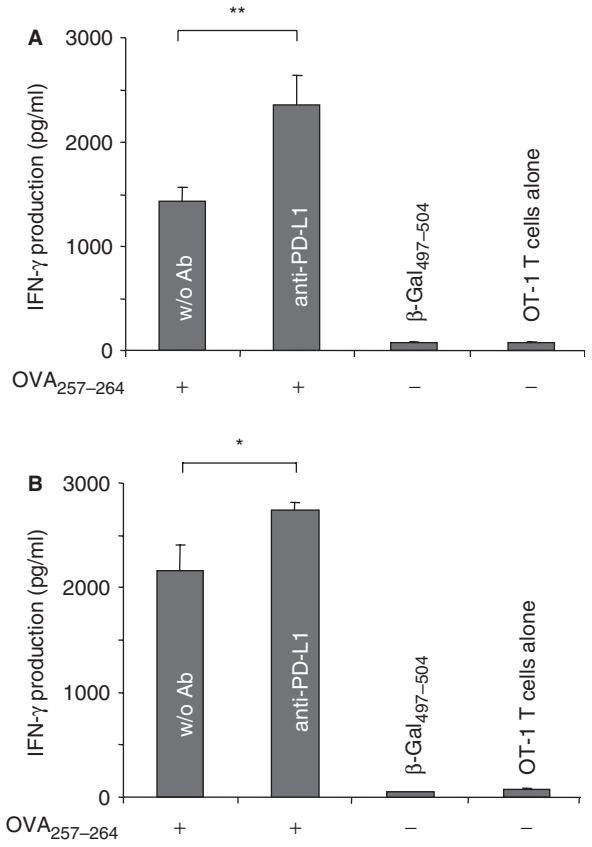

Fig. 6. Blockade of PD-L1 on renal TEC increased OVA peptide-specific OT-1 $CD8^+$ T cell activation. IFN-β (**A**) or IFN-γ (**B**) stimulated, $OVA_{257–264}$ or $β\text{-}Gal_{497–504}$ peptide-pulsed (100 ng/ml) renal B6 TEC (2×10^4 per well) were co-incubated with resting OT-1 $CD8^+$ T cells (1×10^5 per well) in the presence or absence of the PD-L1 blocking mAbs (final concentration 10 μg/ml) for 24 h. OT-1 $CD8^+$ T cell activation was determined by IFN-γ production from the collected supernatants with IFN-γ ELISA. Data represent the mean amount of IFN-γ ± SD. One out of the four independent experiments is shown. $*P \leq 0.05$; $**P \leq 0.001$, when compared with TEC without Ab blockade (Student's *t*-test).

PD-L1 is also an important control molecule in autoimmunity and transplant rejection. Mice that are deficient in PD-L1 and/or PD-1 have a strong tendency to develop autoimmune diseases [28,29]. In disease models such as autoimmune diabetes and experimental autoimmune encephalomyelitis (EAE), upregulated PD-L1 expression has been observed [30–33]. Enhanced expression of PD-L1 has also been reported in allograft transplants of kidney, heart, skin and islets of pancreas, indicating that PD-L1 plays a role in the downregulation of the rejection process [2,34–36]. Finally, PD-L1 is involved in feto-maternal tolerance mechanisms [37]. Together, these data suggest that the PD-L1/PD-1 pathway plays an important role in peripheral and parenchymal immune tolerance.

During inflammation, the tubulointerstitium and/or TEC are exposed to many pro-inflammatory cytokines that are produced by infiltrating immune cells. We have previously reported that IFN-γ treatment led to the strong increase of PD-L1 expression in TEC [2]. An additional important finding in this study is that the type I interferon IFN-β is able to induce strong PD-L1 expression in TEC and that the IFN-β-induced PD-L1 on TEC reduced specific $CD8^+$ CTL responses. IFN-β is the principal immune modulation agent to treat patients with multiple sclerosis (MS) [38–40]. Schwarting *et al.* [41] reported that IFN-β treatment prolonged survival of MRL-Fas^{lpr} mice with experimental systemic lupus erythematosus (SLE) and reduced kidney pathology. The molecular mechanisms of IFN-β treatment on autoimmune diseases still remain unclear, but one hypothesis might be that the administration of IFN-β is able to induce an upregulation of PD-L1 on peripheral professional APC and on non-lymphoid tissues, thereby downregulating T cell proliferation and the infiltration of leukocytes. Indeed, Schreiner *et al.* [40] showed that the therapeutic application of IFN-β leads to a significant increase of PD-L1 level in MS patients, suggesting that PD-L1 might be responsible, at least in part, for the protective effect in this therapy. More detailed studies are required to test this hypothesis

IFN-γ is a pivotal pro-inflammatory cytokine in immune-mediated renal diseases [42]. However, its immunoregulatory effect *in vivo* is still unclear so far. Some studies showed that IFN-γ is essential for the disease development [43,44]; whereas others reported that endogenous IFN-γ plays a protective role in renal organ-specific autoimmunity [45]. Although IFN-γ treatment showed immunoregulatory effect to down-regulate specific CTL responses under our *in vitro* experimental conditions, its *in vivo* role of organ-specific immunomodulation still needs to be elucidated.

The results obtained from the current study were based on an *in vitro* experimental system that used primary TEC cultures and antigen-specific $CD8^+$ CTL from OT-1 transgenic mice to study the co-stimulatory function of epithelial PD-L1. The *in vivo* role of intrarenal PD-L1 on modulating

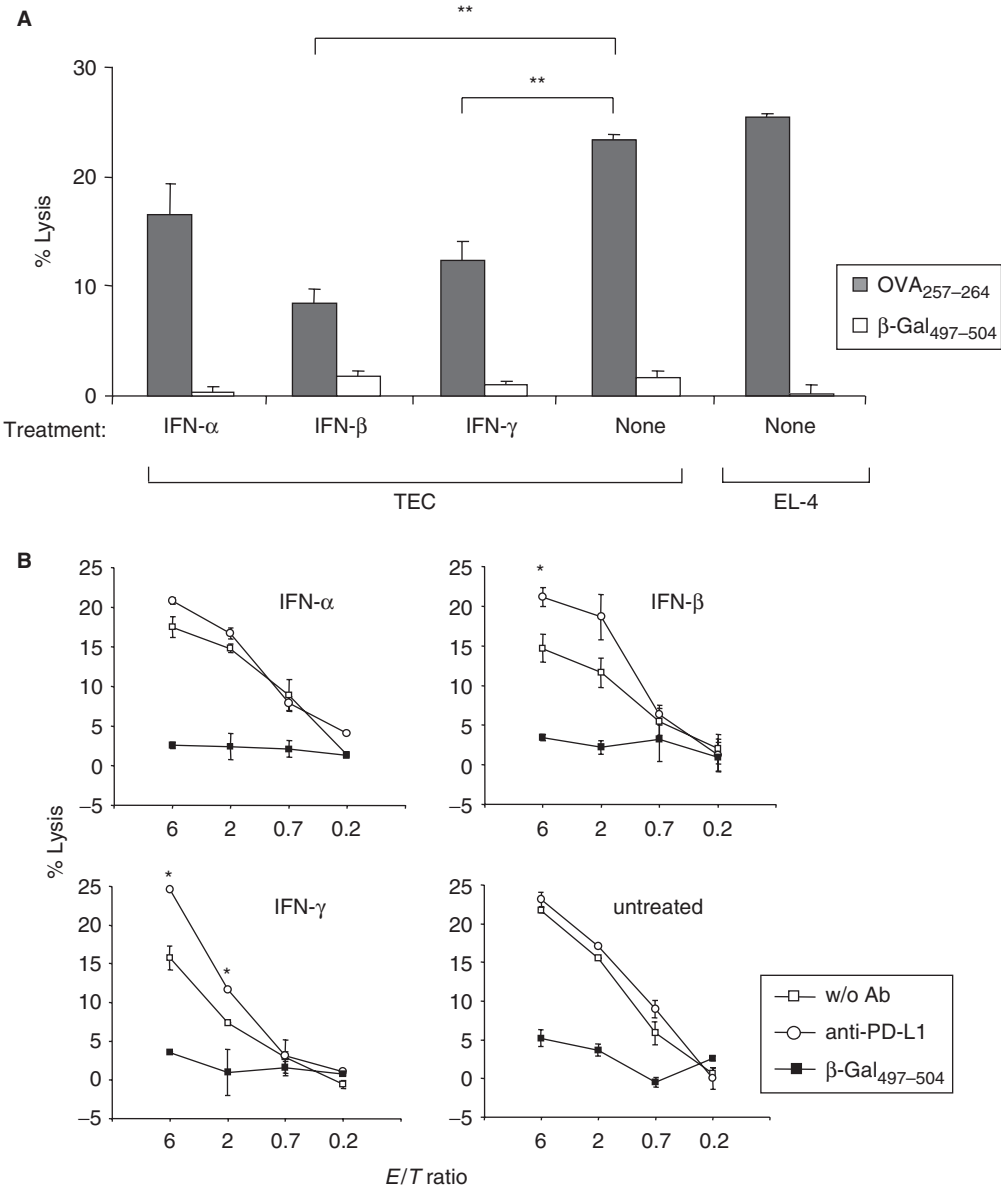

Fig. 7. High PD-L1-expressing TEC were partially protected from cytolysis by activated OT-1 $CD8^+$ CTL. (**A**) IFN-α, IFN-β or IFN-γ treated or untreated renal B6 TEC or EL-4 cells pulsed with 10 ng/ml of $OVA_{257–264}$ or $β\text{-}Gal_{497–504}$ peptide were co-incubated with activated OT-1 $CD8^+$ CTL as an E/T ratio 10:1 (1×10^5 of CTL to 1×10^4 of TEC per well) for CTL assay. After 4 h of incubation, specific cytolysis of targets was measured with the CytoTox 96® non-radioactive cytotoxicity assay and calculated as described in 'Materials and methods'. $^*P \leq 0.05$; $^{**}P \leq 0.001$, when compared with untreated TEC (Student's t-test). (**B**) CTL assay was performed by using IFN-α, IFN-β or IFN-γ pre-treated or untreated, $OVA_{257–264}$ peptide-pulsed B6 TEC as targets as described in (**A**), in the presence or absence of anti-mouse PD-L1 mAb (10 μg/ml). TEC pulsed with $β\text{-}Gal_{497–504}$ peptide was used as control. Data represent the mean % lysis ± SD. One out of three independent experiments is shown. $^*P \leq 0.05$; $^{**}P \leq 0.001$, when compared with TEC without Ab blockade (Student's t-test).

T cell responses requires further verification. Nevertheless, considering the evidence that the *in vivo* PD-L1 expression is markedly enhanced in kidneys with tubulitis in renal allograft rejection [2] and in lupus nephritis [46], and our previous findings that the renal epithelial PD-1/PD-L1 pathway inhibits antigen-specific $CD4^+$ T helper cell activation *in vitro* [2], our data indicated that the renal epithelial PD-L1/PD-1 pathway may inhibit both $CD4^+$ and $CD8^+$ T cell responses and protect the renal epithelial cells from the attack of CTL.

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Conflict of interest statement. None declared.

References

1. van Kooten C, Daha MR, van Es LA. Tubular epithelial cells: a critical cell type in the regulation of renal inflammatory processes. *Exp Nephrol* 1999; 7: 429–437
2. Schoop R, Wahl P, Le Hir M *et al*. Suppressed T-cell activation by IFN-gamma-induced expression of PD-L1 on renal tubular epithelial cells. *Nephrol Dial Transplant* 2004; 19: 2713–2720
3. de Haij S, Woltman AM, Trouw LA *et al*. Renal tubular epithelial cells modulate T-cell responses via ICOS-L and B7-H1. *Kidney Int* 2005; 68: 2091–2102
4. Neilson EG. The nephritogenic T lymphocyte response in interstitial nephritis. *Semin Nephrol* 1993; 13: 496–502
5. Meyers CM, Kelly CJ. Effector mechanisms in organ-specific autoimmunity. I. Characterization of a $CD8^+$ T cell line that mediates murine interstitial nephritis. *J Clin Invest* 1991; 88: 408–416
6. Yamamoto T, Fujinaka H, Kawasaki K *et al*. $CD8^+$ lymphocytes play a central role in the development of anti-GBM nephritis through induction of ICAM-I and chemokines in WKY rats. *Contrib Nephrol* 1996; 118: 109–112
7. Wang Y, Wang YP, Tay YC *et al*. Role of $CD8^+$ cells in the progression of murine adriamycin nephropathy. *Kidney Int* 2001; 59: 941–949
8. Yuan R, El-Asady R, Liu K *et al*. Critical role for $CD103^+CD8^+$ effectors in promoting tubular injury following allogeneic renal transplantation. *J Immunol* 2005; 175: 2868–2879
9. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol* 2002; 2: 116–126
10. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005; 23: 515–548
11. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004; 4: 336–347
12. Ding H, Wu X, Gao W. PD-L1 is expressed by human renal tubular epithelial cells and suppresses T cell cytokine synthesis. *Clin Immunol* 2005; 115: 184–191
13. Hogquist KA, Jameson SC, Heath WR *et al*. T cell receptor antagonist peptides induce positive selection. *Cell* 1994; 76: 17–27
14. Wuthrich RP, Glimcher LH, Yui MA *et al*. MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. *Kidney Int* 1990; 37: 783–792
15. Benz PS, Fan X, Wuthrich RP. Enhanced tubular epithelial CD44 expression in MRL-lpr lupus nephritis. *Kidney Int* 1996; 50: 156–163
16. Wuthrich RP, Sibalic V. Autoimmune tubulointerstitial nephritis: insight from experimental models. *Exp Nephrol* 1998; 6: 288–293
17. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* 2005; 23: 23–68
18. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol Immunother* 2005; 54: 307–314
19. Thompson RH, Gillett MD, Cheville JC *et al*. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci USA* 2004; 101: 17174–17179
20. Dong H, Strome SE, Salomao DR *et al*. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002; 8: 793–800
21. Wintterle S, Schreiner B, Mitsdoerffer M *et al*. Expression of the B7-related molecule B7-H1 by glioma cells: a potential mechanism of immune paralysis. *Cancer Res* 2003; 63: 7462–7467
22. Brown JA, Dorfman DM, Ma FR *et al*. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol* 2003; 170: 1257–1266
23. Saudemont A, Quesnel B. In a model of tumor dormancy, long-term persistent leukemic cells have increased B7-H1 and B7.1 expression and resist CTL-mediated lysis. *Blood* 2004; 104: 2124–2133
24. Hirano F, Kaneko K, Tamura H *et al*. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005; 65: 1089–1096
25. Barber DL, Wherry EJ, Masopust D *et al*. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 2006; 439: 682–687
26. Becker JL, Miller F, Nuovo GJ *et al*. Epstein-Barr virus infection of renal proximal tubule cells: possible role in chronic interstitial nephritis. *J Clin Invest* 1999; 104: 1673–1681
27. Hariharan S. BK virus nephritis after renal transplantation. *Kidney Int* 2006; 69: 655–662
28. Latchman YE, Liang SC, Wu Y *et al*. PD-L1-deficient mice show that PD-L1 on T cells, antigen-presenting cells, and host tissues negatively regulates T cells. *Proc Natl Acad Sci USA* 2004; 101: 10691–10696
29. Nishimura H, Nose M, Hiai H *et al*. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999; 11: 141–151
30. Liang SC, Latchman YE, Buhlmann JE *et al*. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 2003; 33: 2706–2716
31. Ansari MJ, Salama AD, Chitnis T *et al*. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* 2003; 198: 63–69
32. Keir ME, Liang SC, Guleria I *et al*. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 2006; 203: 883–895
33. Salama AD, Chitnis T, Imitola J *et al*. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med* 2003; 198: 71–78
34. Koga N, Suzuki J, Kosuge H *et al*. Blockade of the interaction between PD-1 and PD-L1 accelerates graft arterial disease in cardiac allografts. *Arterioscler Thromb Vasc Biol* 2004; 24: 2057–2062
35. Sandner SE, Clarkson MR, Salama AD *et al*. Role of the programmed death-1 pathway in regulation of alloimmune responses *in vivo*. *J Immunol* 2005; 174: 3408–3415
36. Gao W, Demirci G, Strom TB *et al*. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* 2003; 76: 994–999
37. Guleria I, Khosroshahi A, Ansari MJ *et al*. A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J Exp Med* 2005; 202: 231–237
38. Arnason BG. Interferon beta in multiple sclerosis. *Clin Immunol Immunopathol* 1996; 81: 1–11
39. Yong VW. Differential mechanisms of action of interferon-beta and glatiramer aetate in MS. *Neurology* 2002; 59: 802–808
40. Schreiner B, Mitsdoerffer M, Kieseier BC *et al*. Interferon-beta enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *J Neuroimmunol* 2004; 155: 172–182
41. Schwarting A, Paul K, Tschirner S *et al*. Interferon-beta: a therapeutic for autoimmune lupus in MRL-Faslpr mice. *J Am Soc Nephrol* 2005; 16: 3264–3272
42. Fan X, Wuthrich RP. Upregulation of lymphoid and renal interferon-gamma mRNA in autoimmune MRL-Fas^{lpr} mice with lupus nephritis. *Inflammation* 1997; 21: 105–112
43. Jacob CO, van der Meide PH, McDevitt HO. *In vivo* treatment of (NZB X NZW)F1 lupus-like nephritis with monoclonal antibody to gamma interferon. *J. Exp. Med.* 1987; 166: 798–803

44. Richards HB, Satoh M, Jennette JC *et al.* Interferon-γ is required for lupus nephritis in mice treated with the hydrocarbon oil pristane. *Kidney Int* 2001; 60: 2173–2180
45. Kitching AR, Turner AL, Semple T *et al.* Experimental Autoimmune Anti-Glomerular Basement Membrane Glomerulonephritis: A Protective Role for IFN-γ. *J Am Soc Nephrol* 2004; 15: 1764–1774
46. Chen Y, Zhang J, Li J *et al.* Expression of B7-H1 in inflammatory renal tubular epithelial cells. *Nephron Exp Nephrol* 2006; 102: e81–e92

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CHAPTER 3

TGF-beta treatment modulates PD-L1 and CD40 expression in proximal renal tubular epithelial cells and enhances CD8$^+$ cytotoxic T cell Responses

Astrid Starke, Rudolf P. Wüthrich and Ying Waeckerle-Men

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TGF-Beta Treatment Modulates PD-L1 and CD40 Expression in Proximal Renal Tubular Epithelial Cells and Enhances CD8$^+$ Cytotoxic T-Cell Responses

Astrid Starke[a] Rudolf P. Wüthrich[a, b] Ying Waeckerle-Men[a]

[a]Institute of Physiology and Zürich Center for Integrative Human Physiology, University of Zürich-Irchel, and [b]Clinic for Nephrology, University Hospital Zürich, Zürich, Switzerland

Key Words
Molecules, co-stimulatory · Cytotoxic T cells · Renal proximal tubular epithelial cells · Transforming growth factor-β

Abstract
Background/Aim: TGF-β expression is increased in immune-mediated and fibrotic renal diseases and modulates the tubulointerstitial T-cell response. We examined whether TGF-β changes the expression of PD-L1 and CD40 in the renal proximal tubular epithelial cell (TEC), and whether the activation of CD8$^+$ cytotoxic T cells (CTLs) is influenced by TGF-β treatment of TECs. ***Methods:*** Murine TECs were treated with TGF-β or IFN-γ. The expression of PD-L1 and CD40 was examined with RT-PCR and flow cytometry. To investigate if TGF-β treatment influenced the antigen presentation capacity of TECs, OT-1 CTLs were co-incubated with TGF-β-treated, $OVA_{257–264}$ peptide-pulsed congeneic TECs. The cytotoxicity of OT-1 CTLs was estimated by their capacity to produce IFN-γ and their cytolytic activity. ***Results:*** TGF-β treatment lead to a transition in morphology of renal TECs and downregulated the basal and the IFN-γ-stimulated PD-L1 expression in TECs. Interestingly, TGF-β treatment of TECs increased the constitutive and IFN-γ-induced CD40 expression. In contrast to IFN-γ which reduced the CTL activity, TGF-β treatment of TECs elevated the OVA-specific CTL response. ***Conclusion:*** Our data show that TGF-β changed the cellular phenotype and the expression of PD-L1 and CD40 on TECs and enhanced the activity of OVA peptide-specific CD8$^+$ T cells. TGF-β may thereby play an important role in the progression of renal tubulointerstitial damage in CD8$^+$ T-cell-mediated renal diseases.

Introduction

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine involved in multiple physiological processes such as cell proliferation, wound healing and immune processes [1]. Elevated expression of TGF-β is detected in many renal diseases. It has been shown that TGF-β plays a critical role in renal disorders such as IgA nephropathy, tubulointerstitial injury and acute and chronic renal allograft rejection [2–4], and that blockade of TGF-β is beneficial in experimental kidney diseases [5, 6]. During tubulointerstitial injury, inflammation and fibrosis are key events that may lead to chronic renal failure [7]. TGF-β is the most important regulator of both processes because it acts on the renal epithelium and on infiltrating immune cells. TGF-β induces a process known as epithelial-to-mesenchymal transition (EMT) in renal tubular epithelial cells (TECs) [8–10]. This complex process of

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Dr. Ying Waeckerle-Men
Institute of Physiology, University of Zürich-Irchel
Winterthurerstrasse 190
CH–8057 Zürich (Switzerland)
Tel. +41 44 635 5107, Fax +41 44 635 6814, E-Mail ying.waeckerle-men@access.uzh.ch

transition includes loss of epithelial cell adhesion, disruption of tubular basement membrane and enhanced cell migration and invasion, which promotes renal fibrogenesis [11].

Another function of TGF-β is the regulation of immune responses. It directly affects, among others, proliferation, differentiation and survival of T cells [1]. It has been reported that a high amount of TGF-β promoted the recruitment of allogenic $CD8^+$ T cells and increased the T-cell/TEC contact during acute rejection by upregulation of the CD103 ($\alpha_E\beta_7$ integrin) molecule on $CD8^+$ T cells, which binds to E-cadherin on renal epithelial cells [12].

Due to their expression of MHC class I and II molecules and certain co-stimulatory molecules, renal TECs can behave as 'non-professional' antigen-presenting cells (APCs) and therefore are able to present foreign antigens to $CD4^+$ and $CD8^+$ T cells. The co-stimulatory molecule expression pattern on renal TECs depends on the surrounding cytokine milieu. For example, the proinflammatory cytokine interferon-γ (IFN-γ) enhances MHC class I and II molecules on renal TECs and also increases the expression of co-stimulatory molecules such as programmed death ligand 1 (PD-L1 or B7-H1) and inducible co-stimulator ligand (ICOS-L or B7RP1) [13, 14]. PD-L1, a member of the B7 family, is widely expressed on lymphoid and non-lymphoid cells. Interaction of PD-L1 with its receptor programmed cell death 1 (PD-1) on T and B cells suppresses lymphocyte activation. We have previously reported that the IFN-γ induced upregulation of PD-L1 on renal TECs inhibits antigen-specific T-cell responses in vitro [14, 15].

The CD40-CD40L co-stimulatory pathway may also be critical in the interaction between renal TECs and T cells. CD40 belongs to the family of tumor necrosis factor receptors and is expressed on professional APCs, endothelial cells, fibroblast and epithelial cells. Its ligand CD154 (CD40L) is mainly found on activated T cells. The interaction between both molecules is responsible for the maturation of APCs (e.g. dendritic cells) and the priming of T-cell responses. CD40 is weakly expressed on renal epithelial cells under healthy conditions and is increased during lupus glomerulonephritis, rejection and murine chronic proteinuria [16–18]. The interaction of renal CD40 with CD40L on infiltrating T cells influences the T-cell response as well as the function of renal TECs. On the other hand, the CD40-CD40L pathway is also involved in the development of fibrosis in several organs such as lung and liver [19]. Stimulation of CD40 on fibroblasts by CD40L-bearing T cells and mast cells leads to proliferation of fibroblasts, production of proinflammatory mediators and extracellular matrix components by fibroblasts [19]. Pontrelli et al. [20] showed that ligation of CD40 on renal TECs has not only proinflammatory but also profibrotic effects.

Despite its well-defined role in renal fibrosis, the function of TGF-β on the regulation of co-stimulatory and co-inhibitory molecule expression on renal TEC is largely unknown. Thus, we investigated the effect of TGF-β on the expression of the co-inhibitory molecule PD-L1 and on the co-stimulatory molecule CD40 on murine primary TECs and TEC cell line C1.1. TGF-β-treated TECs were exposed to antigen-specific OT-1 $CD8^+$ T cells to explore their cytotoxic response. The effects of TGF-β stimulation on renal TECs were compared to the influences of its antagonistic cytokine IFN-γ which is a potent inducer of PD-L1 but counteracts the profibrotic action of TGF-β on renal TECs [21].

Methods

General Reagents

Cell culture reagents were obtained from Invitrogen (Gaithersburg, Md., USA) and Sigma (St. Louis, Mo., USA). Recombinant mouse IFN-γ and human TGF-β were purchased from R&D Systems (Oxford, UK). Anti-mouse PD-L1 and CD40 monoclonal antibodies (mAbs) were obtained from eBioscience (San Diego, Calif., USA). Biotin-conjugated anti-mouse $H\text{-}2K^k$ was obtained from BD Biosciences (San Jose, Calif., USA). Chicken ovalbumin peptide 257–264 ($OVA_{257–264}$, amino acid sequence SIINFEKL) was purchased from Proimmune (London, UK). An $H\text{-}2K^b$ restricted β-galactosidase peptide 497–504 ($\beta\text{-}Gal_{497–504}$, amino acid sequence ICPMYARV) was used as a control. Anti-mouse CD8a (Ly-2) MACS microbeads were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany).

Animals and Cell Cultures

OT-1 mice [22] were obtained from the Jackson Laboratory (Bar Harbor, Me., USA) and C57BL/6 (B6) mice were purchased from Harlan (Horst, the Nertherlands). All animals were maintained in the animal facility at the Irchel Campus of the University of Zürich, Switzerland. The study protocol was approved by the regulatory commission for animal studies of the Canton of Zürich, Switzerland. Primary cultures of murine renal proximal TECs and a SV40-transformed murine renal proximal TEC line C1.1 were prepared and cultured as described [23].

RT-PCR Analysis

Total RNA was extracted from the indicated cell cultures with RNeasy® mini kit (Qiagen, Valencia, Calif., USA). All samples were quantified by the measurement of the optical density at 260 nm, and 1 μg of RNA was amplified using OneStep RT-PCR kit (Qiagen). Primer sequences of PD-L1 and CD40 were determined as previously described [14]. PD-L1 and CD40 mRNA levels were

normalized by the corresponding GAPDH mRNA levels. Reaction mixtures were separated on 1.5% agarose gels containing ethidium bromide, and bands were detected under UV light and analyzed with the Bio-Image System Chemidoc™ XRS (Bio-Rad, Hercules, Calif., USA). Densitometry analysis was performed with the Quantity One Software 4.6.1 (Bio-Rad Laboratories, Inc).

Flow Cytometry Analysis

The TEC line C1.1 and primary cultures of renal TECs were stimulated with different concentrations of TGF-β alone or in combination with IFN-γ for 48 h. Cells were harvested by light trypsinization, washed and suspended in PBS containing 2% FBS and 0.1% sodium azide. After incubating with primary mAbs for 45 min on ice, cells were washed twice and incubated with the appropriate FITC-conjugated secondary Ab for 30 min. Cells were then washed and analyzed using a FACScan flow cytometer and the Cell Quest™ software (Becton Dickinson).

Determination of Morphology of TECs

Primary renal TECs were treated either with TGF-β or IFN-γ for 6 and 48 h. The morphology of TECs was determined with an Axiovert 200M microscope (Carl Zeiss AG, Germany) and analyzed with AxioVision software (Carl Zeiss AG, Germany).

Isolation of CD8$^+$ T Cells from OT-1 Mice

Spleen and lymph nodes were harvested from 8- to 16-week-old naive OT-1 mice. CD8$^+$ cells were isolated using anti-mouse CD8a (Ly-2) MACS microbeads according to the manufacturer's protocol. The freshly isolated OT-1 CD8$^+$ T cells were either used immediately for antigen presentation assays or were activated in vitro by stimulation with B6 splenocytes pulsed with 0.1 μg/ml of $OVA_{257–264}$ peptide. Cells were incubated in DMEM medium supplemented with 5 μM 2-mercaptomethanol, 1% HEPES, 1,000 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. After 6 days of stimulation, activated OT-1 CTLs were used for CTL assays.

Antigen Presentation of Renal B6 TECs to OT-1 CD8$^+$ T Cells

To study the antigen presentation of the H-2K^b-restricted peptide $OVA_{257–264}$ by congeneic primary TECs to OT-1 CD8$^+$ T cells, B6 primary TECs were pretreated with IFN-γ (100 U/ml) or TGF-β (2 ng/ml) for 48 h. Cells were washed to remove excess cytokines and were harvested by light trypsinization. The cells were then pulsed with 0.1 μg/ml of $OVA_{257–264}$ peptide or the control H-2K^b-restricted β-$Gal_{497–504}$ peptide for 1 h at 37°C. After washing, TECs were counted and seeded in 96-well U-bottomed cell culture plates (2×10^4/well) in DMEM medium supplemented with 5% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The resting OT-1 CD8$^+$ T cells were added to TEC cultures (2×10^5/well) and were co-incubated at 37°C. After 24 h, supernatants from co-cultures were collected and analyzed for IFN-γ content using a mouse IFN-γ-specific ELISA kit (OptEIA™ mouse IFN-γ kit, BD Pharmingen).

Cytotoxicity by Activated OT-1 CD8$^+$ CTLs to B6 TECs

To measure the antigen-specific cytolysis of renal TECs by CTLs, activated OT-1 CD8$^+$ CTLs were added to TGF-β- or IFN-γ-treated $OVA_{257–264}$ peptide-loaded B6 TECs in 96 U-bottomed cell culture plates at effector/target ratios of 10:1 or 5:1 as indicated. After 4 h of incubation, 50 μl of supernatants were col-

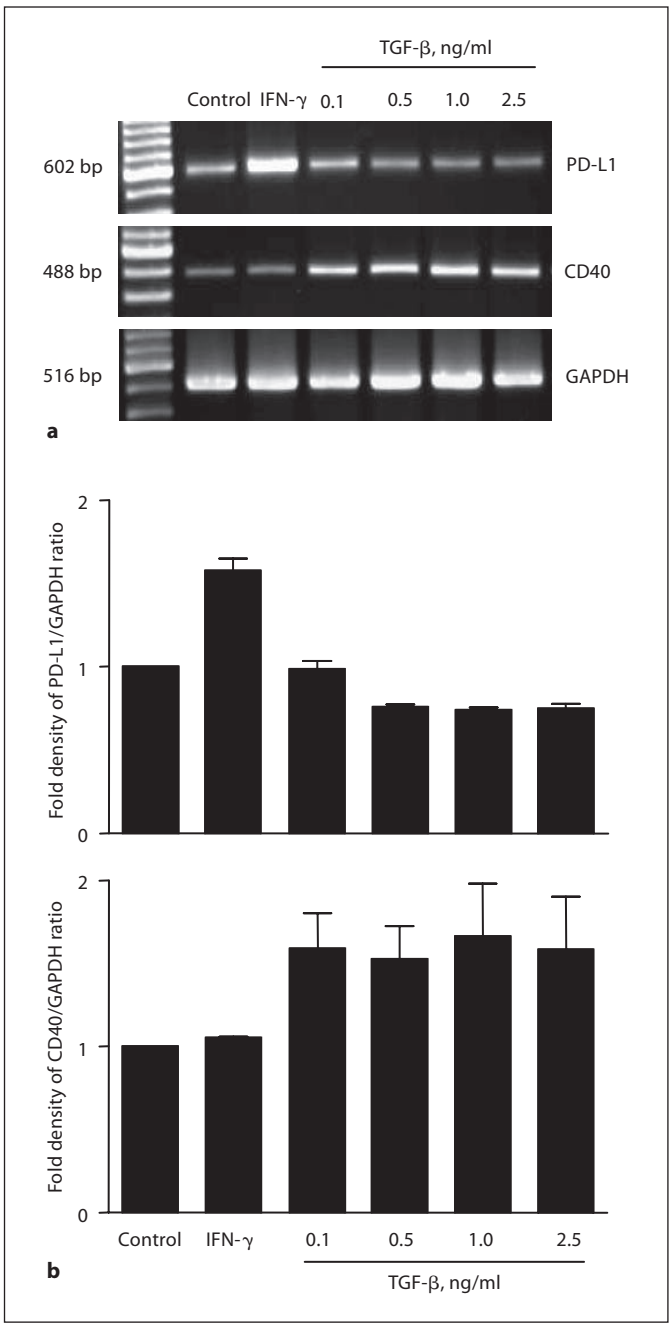

Fig. 1. RT-PCR analysis of PD-L1 and CD40 mRNA expression in renal TEC after treatment with TGF-β. **a** Agarose gel stained with ethidium-bromide. C1.1 cells were treated with different concentrations of TGF-β or IFN-γ (100 U/ml) for 6 h or without cytokine treatment. GAPDH was used as a housekeeping gene. **b** Densitometry analysis of the agarose gel showed in **a** was performed. The density of the PD-L1/ GAPDH ratio was calculated as the fold density of the untreated mRNA level of C1.1 cells. Data represent the mean ratio of each sample ± SD from two independent loadings of the same RT-PCR sample.

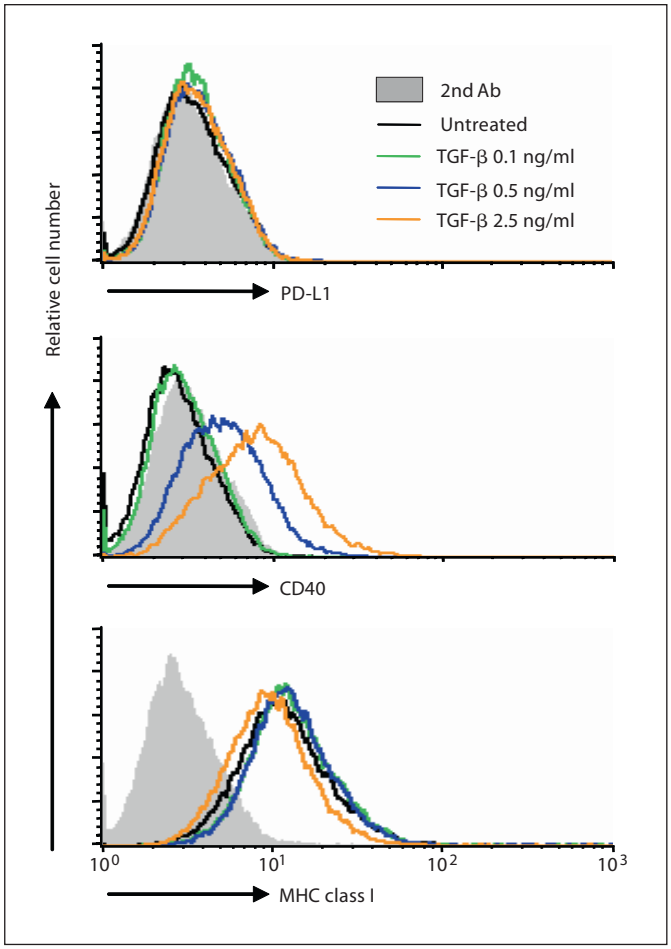

Fig. 2. Expression of surface PD-L1, CD40 and MHC class I molecules on renal TECs after TFG-β treatment. C1.1 cells stimulated with different concentrations of TGF-β for 48 h or without treatment were stained with anti-mouse PD-L1, CD40 and MHC class I mAbs for flow cytometric analysis. Results are representative of 3 independent experiments.

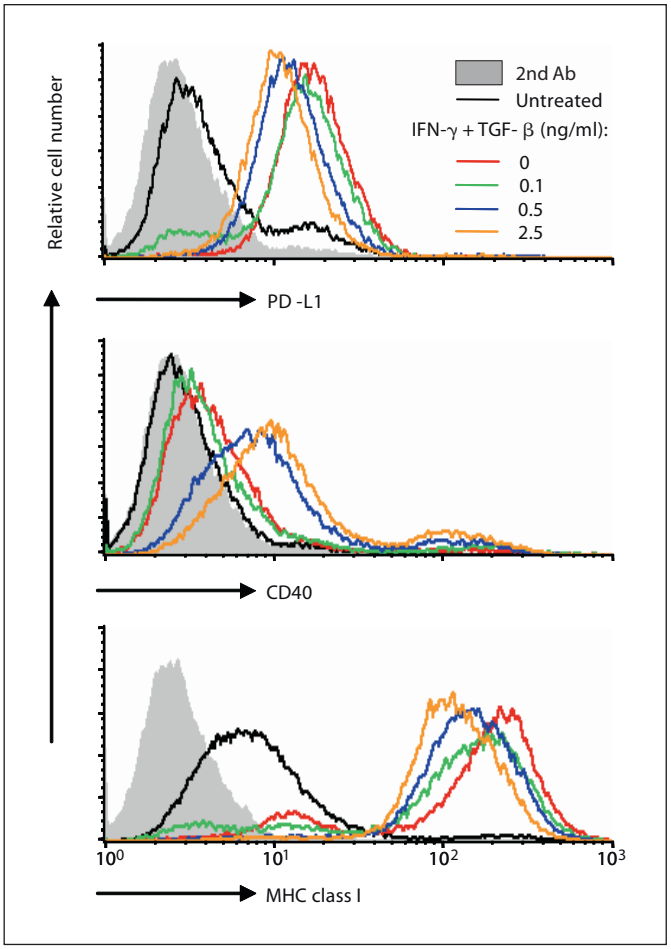

Fig. 3. TGF-β altered the IFN-γ-induced surface expression of PD-L1, CD40 and MHC class I molecules on renal TECs. C1.1 cells were stimulated with different concentrations of TGF-β for 48 h in the presence of IFN-γ (100 U/ml) or without treatment. Cells were then stained with anti-mouse PD-L1, CD40 and MHC class I mAbs for flow cytometric analysis. Results are representative of 3 independent experiments.

lected from each well to measure the amount of lactate dehydrogenase (LDH) using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega) according to the manufacturer's protocol. The percentage of target lysis was calculated as: % lysis = 100 × (experimental LDH release – spontaneous LDH release of targets – spontaneous LDH release from T cells)/(maximum LDH release – spontaneous LDH release of targets).

Statistics

All experiments were performed at least three times. Results from IFN-γ ELISA or CTL assays are expressed as means ± SD of triplicate determinations from the representative experiments that gave rise to similar results. Statistical analysis was performed using unpaired Student's t test. Significance was accepted at $p \leq 0.05$.

Results

TGF-β Treatment Altered the Expression of PD-L1, CD40 and MHC Class I Molecules in Renal TECs

The TEC line C1.1 and primary TECs were stimulated with different concentrations of TGF-β to determine the mRNA and surface expression of PD-L1, CD40 and MHC class I molecules. Untreated and IFN-γ-stimulated TECs were used as controls. Figure 1 shows that constitutive PD-L1 and CD40 mRNAs were detected in C1.1 cells and primary TECs without cytokine stimulation. IFN-γ treatment led to the increase in PD-L1 mRNA, whereas only a slight change in CD40 mRNA

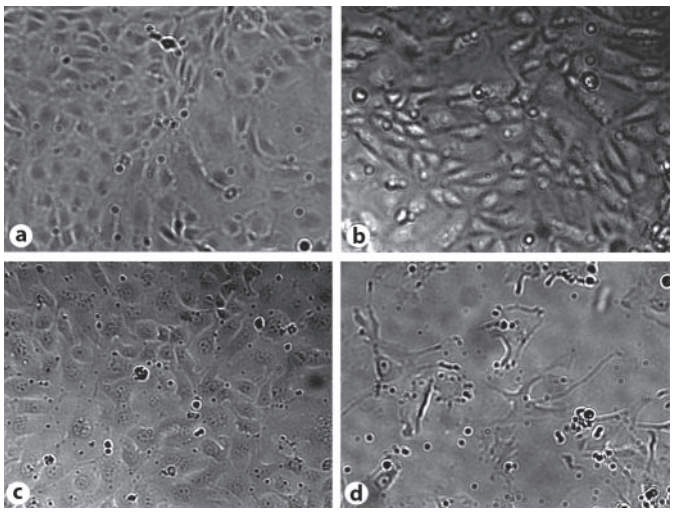

Fig. 4. Representative photomicrographs of murine B6 primary renal TECs after stimulation with IFN-γ (100 U/ml) or TGF-β (2.5 ng/ml) for 6 or 48 h. **a** Untreated. **b** IFN-γ 48 h. **c** TGF-β 6 h. **d** TGF-β 48 h. ×20.

was observed with IFN-γ treatment. A decrease in PD-L1 mRNA was observed after TGF-β stimulation at concentrations of 0.5–2.5 ng/ml. Interestingly, treatment with TGF-β strongly enhanced the CD40 mRNA level in renal TECs (fig. 1). Flow cytometry analysis revealed a strong increase in CD40 on the renal TEC surface after TGF-β treatment but no surface expression of PD-L1 on TECs could be detected after TGF-β treatment (fig. 2). In addition, TGF-β treatment slightly downregulated the constitutive MHC class I expression on renal TECs.

TGF-β Downregulated IFN-γ-Induced Upregulation of PD-L1 and MHC Class I Molecules and Increased CD40 Expression

To determine whether inflammatory-mediated upregulation of PD-L1 and CD40 can be altered by TGF-β treatment, renal TECs were treated with TGF-β in the presence of IFN-γ. As shown in figure 3, TGF-β stimulation downregulated the IFN-γ-induced upregulation of PD-L1 and MHC class I molecules in a dose-dependent manner. Very interestingly, TGF-β treatment further increased the IFN-γ-induced CD40 upregulation on renal TECs (fig. 3). These results indicated that TGF-β is a modulator of IFN-γ-induced PD-L1 and MHC class I molecule expression and a strong stimulus to increase CD40 expression on renal TECs.

TGF-β Treatment Induced a Change in Morphology in Renal TECs

TGF-β is the main inducer of EMT in renal epithelial cells [8]. To show that TGF-β was also profibrotic in our cell culture system, primary renal TECs were treated with TGF-β or IFN-γ. As shown in figure 4, untreated renal TECs were closely attached to each other and displayed a normal cobble-stone morphology (fig. 4a). Stimulation with IFN-γ for 48 h did not change the morphology of renal TECs (fig. 4b). Although there was no visible change in renal TECs after treatment with TGF-β for 6 h (fig. 4c), alterations in the morphology of TECs were observed within 24 h (data not shown). The 48-hour incubation with TGF-β induced a remarked morphological change in renal TECs (fig. 4d). The confluent monolayer was disrupted as the cells detached from the bottom of the cell dish. The cells became elongated, formed filopodia and showed a spindle-shaped, fibroblastic morphology. Since the change in co-stimulatory molecule expression reached a plateau level at 48 h, we used this time point for the subsequent experiments.

TGF-β Stimulation of B6 TECs Enhanced Their Capacity to Activate Naive OT-1 CD8$^+$ T Cells

To determine if the antigen-presenting capacity of renal B6 TECs to $OVA_{257–264}$ peptide-specific OT-1 CD8$^+$ T cells was influenced by TGF-β stimulation of TECs, renal B6 TECs were pretreated with TGF-β or IFN-γ and loaded with $OVA_{257–264}$ peptide or the irrelevant peptide β-$Gal_{497–504}$. The activation of naive OT-1 CD8$^+$ T cells was measured by their IFN-γ production. The antigen-presenting capacity of TGF-β-stimulated TECs differed from the ability of untreated and IFN-γ-treated TECs (fig. 5a). A significant increase in IFN-γ was measured after TGF-β stimulation of TECs compared to untreated and IFN-γ treated TECs. IFN-γ-treated TECs even showed a reduced capacity to stimulate OT-1 CD8$^+$ T cells to produce IFN-γ compared to untreated TECs. TECs loaded with the irrelevant peptide β-$Gal_{497–504}$ failed to induce production of IFN-γ by OT-1 CD8$^+$ T cells. Thus, TGF-β stimulation of renal TECs influenced their capacity to activate antigen-specific OT-1 CD8$^+$ T cells.

TGF-β-Treated B6 TECs Were More Susceptible to Be Killed by Activated OT-1 CD8$^+$ T Cells

Next we examined the effect of TGF-β-stimulated renal TECs on the cytotoxicity of activated OT-1 CD8$^+$ T cells. For this purpose, activated OT-1 CD8$^+$ T cells were incubated with renal B6 TECs pretreated with TGF-β

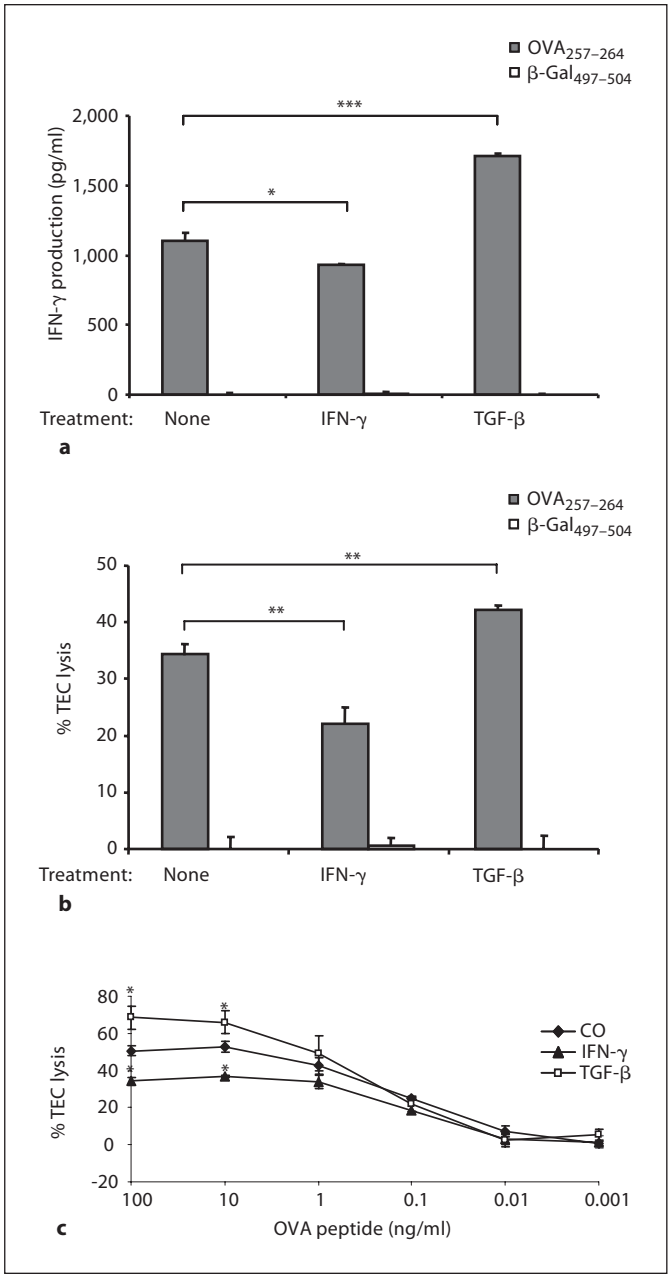

Fig. 5. Higher $OVA_{257–264}$ specific OT-1 $CD8^+$ T-cell responses were induced by TGF-β-treatment of B6 renal TECs. B6 renal TECs were pretreated with TGF-β (2 ng/ml), IFN-γ (100 U/ml) for 48 h or untreated. TECs were then loaded with 0.1 μg/ml $OVA_{257–264}$ or the same concentration of an irrelevant β-$Gal_{497–504}$ peptide and incubated with naive OT-1 $CD8^+$ T cells for 24 h to determine the IFN-γ production in the supernatants with ELISA (**a**) or with activated OT-1 $CD8^+$ T cells for 4 h to measure the cytolysis of the targets (**b**). In another experimental setting, different concentrations of $OVA_{257–264}$ peptide were pulsed with renal TECs pretreated as described above and were incubated with activated OT-1 $CD8^+$ T cells (effector/target ratio 5:1) for the cytotoxic assay (**c**). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ Student's t test.

or IFN-γ and loaded with $OVA_{257–264}$ peptide or β-$Gal_{497–504}$ to measure the lysis of TECs. The cytotoxicity of OVA peptide-specific OT-1 $CD8^+$ T cells to TGF-β-treated renal B6 TECs was significantly increased at peptide concentrations of 100 and 10 ng/ml compared to the untreated and IFN-γ treated TECs (fig. 5b, c). IFN-γ-stimulated renal TECs were partially protected from the lysis induced by OT-1 $CD8^+$ cytotoxic T cells. OT-1 $CD8^+$ T cells did not kill B6 TECs incubated with the irrelevant peptide β-$Gal_{497–504}$. In summary, these data show that the OT-1 $CD8^+$ T-cell response was antigen-specific and that TGF-β-treated TECs significantly enhanced the OT-1 $CD8^+$ activity.

Discussion

It is well known that TGF-β is widely involved in different kinds of renal diseases [5, 24] and that it elicits renal fibrogenesis by inducing EMT in renal epithelial cells [8, 9]. However, the effect of TGF-β on co-stimulatory molecule expression by renal TECs and its implications in TEC/$CD8^+$ T-cell interaction has so far not been fully investigated. In the present study, we show that, beside the transition in morphology of renal TECs, in vitro stimulation of renal TECs with TGF-β downregulated the constitutive PD-L1 mRNAs and the IFN-γ-induced PD-L1 surface expression. In contrast, CD40 mRNA and surface protein were clearly enhanced after TGF-β treatment. In addition, the antigen-specific OT-1 $CD8^+$ T-cell activation was increased by using TGF-β-treated TECs as antigen-presenting cells/targets. Thus, in addition of a critical profibrotic factor, TGF-β also affects co-stimulatory molecule expression on renal TECs under our in vitro experimental conditions.

Our study complements former studies that investigated the effect of TGF-β on co-stimulatory molecule expression on renal TECs. Banu and Meyers [25] showed that TGF-β downregulated IFN-γ-induced expression of MHC class II and B7.1 on primary murine renal TECs. In that case, TGF-β stimulation of TECs decreased their antigen-presenting capacity and lowered the production of IL-4 by a $CD4^+$ Th2 clone. In agreement with that study, we also observed that TGF-β treatment reduced the constitutive and IFN-γ-induced MHC class I (and PD-L1) expression on renal TECs. However, our results that TGF-β-treated TECs increased OT-1 $CD8^+$ T-cell responses are controversial to their findings. This difference may be due to the different T cell types used ($CD4^+$ vs. $CD8^+$ T cells).

Co-stimulatory/co-inhibitory signals effectively regulate T-cell activation and tolerance. It is well accepted that the CD40/CD40L co-stimulatory pathway provides a positive signal to increase T-cell responses [26], and the CD40/CD40L interaction is thought to be involved in the regulation of interstitial infiltration in the kidney [18, 27]. Rogers et al. [28] reported that renal TECs expressing CD40 were capable of stimulating the proliferation of $CD4^+$ T cells. On the other hand, the PD-L1/PD-1 pathway provides the negative signal to downregulate $CD4^+$ and $CD8^+$ T-cell responses [14, 15]. The downregulation of PD-L1 and upregulation of CD40 on renal TECs by TGF-β treatment observed in the current study therefore may explain the increased OT-1 $CD8^+$ T-cell activation. As shown previously, TECs with high PD-L1 surface expression were protected from killing by OT-1 $CD8^+$ T cells [15]. In addition, we also observed that blocking CD40 on the TEC surface by anti-mouse CD40 mAbs slightly decreased OT-1 $CD8^+$ T-cell responses (data not shown), although the change was not statistically significant compared with the untreated TECs. Our hypotheses for this phenomenon might be that the CD40 expression on TECs plays a role in fibrosis initiation (discussed below). Another possibility is that the CD40/CD40L co-stimulatory pathway would be more crucial for the activation of $CD4^+$ T cells than for $CD8^+$ T cells [29]. Further studies may be needed to clarify whether TGF-β-treated renal TECs would also be able to increase the $CD4^+$ T-cell activity.

The functional role of renal TEC-expressed CD40 is still not fully understood. Despite its well-know co-stimulatory function on professional APCs, tissue-expressing CD40 is involved in the development of fibrosis [19]. Pontrelli et al. [20] showed that soluble CD40L played a key role in interstitial fibrosis by increasing the expression of the profibrotic peptide plasminogen activator inhibitor-1 in human TECs in vitro. Another study demonstrated that blockade of CD40/CD40L interactions in murine adriamycin nephrosis attenuated not only the interstitial inflammation but also the structural injury [18]. Taken together, we assume that the TGF-β-increased CD40 expression on TECs may function not only as a co-stimulatory molecule but also be involved in amplifying the TGF-β-induced fibrogenesis in the kidney.

In summary, we show that TGF-β not only induced structural alterations but also regulated co-stimulatory molecule expression on renal proximal epithelial cells with functional consequences on their antigen-presenting capacity. Besides its crucial and well-defined influence on renal fibrosis, TGF-β may additionally play an important role in the progression of renal tubulointerstitial damage in $CD8^+$ T-cell-mediated renal diseases.

Acknowledgement

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References

1 Li MO, Wan YY, Sanjabi S, Robertson A-KL, Flavell RA: Transforming growth factor-β; Regulation of immune responses. Annu Rev Immunol 2006;24:99–146.
2 Rastaldi M, Tunesi S, Ferrario F, Indaco A, Zou H, Napodano P, D'Amico G: Transforming growth factor-β, endothelin-1, and c-fos expression in necrotizing/crescentic IgA glomerulonephritis. Nephrol Dial Transplant 1998;13:1668–1674.
3 Danilewicz M, Wagrowska-Danilewicz M: Correlative insights into the immunoexpression of transforming growth factor β-1 in acutely rejected renal allografts. Pathol Res Pract 2006;202:9–15.
4 Pribylova-Hribova P, Kotsch K, Lodererova A, Viklicky O, Vitko S, Volk HD, Lacha J: TGF-β1 mRNA upregulation influences chronic renal allograft dysfunction. Kidney Int 2006;69:1872–1879.
5 Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, Ruoslahti E: Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature 1992;360:361–364.
6 Gagliardini E, Benigni A: Role of anti-TGF-β antibodies in the treatment of renal injury. Cytokine Growth Factor Rev 2006;17:89.
7 Becker GJ, Hewitson TD: The role of tubulointerstitial injury in chronic renal failure. Curr Opin Nephrol Hypertens 2000;9:133–138.
8 Fan J-M, Ng Y-Y, Hill PA, Nikolic-Paterson DJ, Mu W, Atkins RC, Lan HY: Transforming growth factor-β regulates tubular epithelial-myofibroblast transdifferentiation in vitro. Kidney Int 1999;56:1455–1467.
9 Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, Strutz F, Muller GA, Colasanti G, D'Amico G: Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. Kidney Int 2002;62:137–146.
10 Strutz F, Zeisberg M: Renal fibroblasts and myofibroblasts in chronic kidney disease. J Am Soc Nephrol 2006;17:2992–2998.
11 Yang J, Liu Y: Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. Am J Pathol 2001;159:1465–1475.
12 Wong WK, Robertson H, Carroll HP, Ali S, Kirby JA: Tubulitis in renal allograft rejection: role of transforming growth factor-β and interleukin-15 in development and maintenance of $CD103^+$ intraepithelial T cells. Transplantation 2003;75:505–514.

13 Wahl P, Schoop R, Bilic G, Neuweiler J, Le Hir M, Yoshinaga SK, Wuthrich RP: Renal tubular epithelial expression of the costimulatory molecule B7RP-1 (inducible costimulator ligand). J Am Soc Nephrol 2002;13:1517–1526.
14 Schoop R, Wahl P, Le Hir M, Heemann U, Wang M, Wuthrich RP: Suppressed T-cell activation by IFN-γ-induced expression of PD-L1 on renal tubular epithelial cells. Nephrol Dial Transplant 2004;19:2713–2720.
15 Waeckerle-Men Y, Starke A, Wuthrich RP: PD-L1 partially protects renal tubular epithelial cells from the attack of CD8+ cytotoxic T cells. Nephrol Dial Transplant 2007;22:1527–1536.
16 Yellin M, D'Agati V, Parkinson G, Han A, Szema A, Baum D, Estes D, Szabolcs M, Chess L: Immunohistologic analysis of renal CD40 and CD40L expression in lupus nephritis and other glomerulonephritides. Arthritis Rheum 1997;40:124–134.
17 Woltman AM, De Haij S, Boonstra JG, Gobin SJP, Daha MR, Kooten CV: Interleukin-17 and CD40-ligand synergistically enhance cytokine and chemokine production by renal epithelial cells. J Am Soc Nephrol 2000;11:2044–2055.
18 Kairaitis L, Wang Y, Zheng L, Tay Y-C, Wang Y, Harris DCH: Blockade of CD40-CD40 ligand protects against renal injury in chronic proteinuric renal disease. Kidney Int 2003;64:1265–1272.
19 Sime PJ, O'Reilly KMA: Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. Clin Immunol 2001;99:308–319.
20 Pontrelli P, Ursi M, Ranieri E, Capobianco C, Schena FP, Gesualdo L, Grandaliano G: CD40L proinflammatory and profibrotic effects on proximal tubular epithelial cells: role of NF-κB and Lyn. J Am Soc Nephrol 2006;17:627–636.
21 Giannopoulou M, Iszkula SC, Dai C, Tan X, Yang J, Michalopoulos GK, Liu Y: Distinctive role of Stat3 and Erk-1/2 activation in mediating interferon-γ inhibition of TGF-β1 action. Am J Physiol Renal Physiol 2006;290:F1234–F1240.
22 Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR: T cell receptor antagonist peptides induce positive selection. Cell 1994;76:17–27.
23 Wuthrich R, Glimcher L, Yui M, Jevnikar A, Dumas S, Kelley V: MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. Kidney Int 1990;37:783–792.
24 Meyers CM: New insights into the pathogenesis of interstitial nephritis. Curr Opin Nephrol Hypertens 1999;8:287–292.
25 Banu N, Meyers CM: TGF-β1 down-regulates induced expression of both class II MHC and B7–1 on primary murine renal tubular epithelial cells. Kidney Int 1999;56:985–994.
26 Peng X, Kasran A, Warmerdam P, de Boer M, Ceuppens J: Accessory signaling by CD40 for T cell activation: induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. Eur J Immunol 1996;26:1621–1627.
27 van Kooten C, Gerritsma J, Paape M, van Es L, Banchereau J, Daha M: Possible role for CD40-CD40L in the regulation of interstitial infiltration in the kidney. Kidney Int 1997;51:711–721.
28 Rogers NJ, Jackson IM, Jordan WJ, Lombardi G, Delikouras A, Lechler RI: CD40 can costimulate human memory T cells and favors IL-10 secretion. Eur J Immunol 2003;33:1094–1104.
29 Jones ND, Van Maurik A, Hara M, Spriewald BM, Witzke O, Morris PJ, Wood KJ: CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. J Immunol 2000;165:1111–1118.

CHAPTER 4

Establishment of an antigen-specific model of tubulointerstitial nephritis

Astrid Starke[1], Stephan Segerer[2, 3], Michel Le Hir[3], Miyuki Azuma[4], Thomas Fehr[2], Rudolf P. Wüthrich[1, 2] and Ying Waeckerle-Men[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology, University of Zürich, Switzerland;
[2]Division of Nephrology, University Hospital Zürich, Switzerland
[3]Institute of Anatomy, University of Zürich, Switzerland
[4]Tokyo Medical and Dental University, Japan

4.1. Summary

The *in vivo* role of the co-inhibitory molecule PD-L1 in modulating T cell responses in TIN has not been elucidated yet. To investigate the role of PD-L1 in TEC/T cell interactions *in vivo*, we attempted to establish an antigen-specific model of TIN in RIP-mOVA transgenic mice, which express the ovalbumin (OVA) antigen in the pancreas and the kidney. To induce inflammation and T cell infiltration in kidneys of RIP-mOVA mice, two experimental strategies were tested. The first one was to directly transfer activated OVA peptide-specific OT-1 $CD8^+$ T cells (CTLs) into RIP-mOVA mice (CTL transfer strategy). These OVA-specific $CD8^+$ T cells should attack their targets, including OVA-expressing TECs, to induce a renal injury. In the second strategy RIP-mOVA mice were immunized with OVA protein and peptide to induce OVA-specific T cell responses *in vivo* (immunization strategy) against the local "self"-antigen OVA in the kidney and in the pancreas of RIP-mOVA mice.

Diabetes occurred as a consequence of pancreatic islets injury in 70-100% of the RIP-mOVA mice that were injected with activated CTLs. Both adoptive CTL transfer and the immunization strategies induced a marked upregulation of MHC class II and PD-L1 expression on renal TECs in RIP-mOVA mice. The transfer of high amounts of OVA-specific OT-1 CTLs ($10x10^6$) resulted in a rapid and moderate $CD8^+$ T cell accumulation in the renal cortex within 5 days. In contrast, a mild T cell infiltration in the cortical tubulointerstitial space was detected in mice immunized with OVA antigens after two and three weeks. In addition, re-stimulation of T cells isolated from spleens of immunized RIP-mOVA mice *in vitro* gave rise to OVA antigen-specific $CD4^+$ and $CD8^+$ T cell responses, indicating that the immunization with "self-antigens" did not induce an immune tolerance. After adoptive transfer of OVA-specific CTLs anti-PD-L1 monoclonal antibodies were administrated into the mice to try to inhibit the PD-1/PD-L1 pathway *in vivo*. However, no significant influence on the onset of diabetes and kidney infiltration has been observed in the current experimental setting.

Taken together, both strategies induced kidney inflammation, and the PD-1/PD-L1 pathway was activated in the antigen-specific RIP-mOVA model. RIP-mOVA mice represent a useful model to investigate the expression profile and the role of molecules induced on TECs during inflammatory processes in the kidney.

4.2. Introduction

PD-L1 on parenchymal tissues plays a protective role in limiting auto-reactive T cell responses, as already described in the introduction section 3.2. We have shown in several studies that PD-L1 is expressed on murine TECs *in vitro* and *in vivo* and that the enhancement of the PD-1/PD-L1 pathway leads to the inhibition of $CD4^+$ and $CD8^+$ T cell responses *in vitro* (Chapter 1-3, (1)). However, the *in vivo* role of renal tubular epithelial PD-L1 in modulating T cell responses in TIN has not been explored yet. Since there are not many animal models of TIN to study antigen-specific T cell-mediated nephritis, we attempted to establish an antigen-specific model of TIN using RIP-mOVA transgenic mice.

RIP-mOVA mice are on a C57BL/6 (H2-K^b) background and express a membrane-bound form of ovalbumin (mOVA) consisting of the residues 139-385 from mature OVA which is linked to the cytoplasmic and signal/anchor domain of the human transferrin receptor. The cDNA encoding the mOVA fusion protein is under the control of the rat insulin promoter (RIP). RIP-mOVA mice express mOVA in pancreatic β cells, kidney proximal tubular cells (Figure 1) and to a lower extent in the testes and the thymus (2). It has previously been reported that the transfer of OVA-peptide specific $CD8^+$ T cells into these mice induces antigen-specific tissue injury in the pancreas and the kidney.

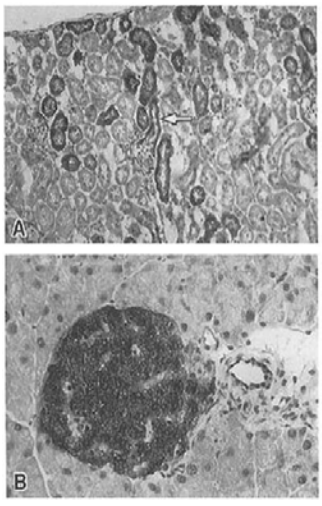

Figure 1: Expression of OVA in RIP-mOVA mice. Immunohistochemistry performed on (A) kidney and (B) pancreas sections. The arrow in A indicates a proximal tubule originating from a glomerulus. Figure and legend are taken from *J Exp Med 184: 923-930, 1996* (2).

The OVA-specific $CD8^+$ T cells used in these experiments are derived from T cell receptor (TCR) transgenic OT-1 (OVA-specific, class I-restricted TCR transgenic) mice. This TCR recognizes the ovalbumin residues 257-264 (SIINFEKL) in the context of H2-K^b (C57BL/6

background). OT-1 mice exhibit the same amount of total T cells as non-transgenic littermates but OT-1 T cells are shifted towards the $CD8^+$ subset (3).

The adoptive transfer of $5x10^6$ naïve OVA-peptide specific OT-1 $CD8^+$ T cells into RIP-mOVA mice leads to the homing, accumulation and activation of these cells in the draining lymphnodes (LNs) of the OVA-expressing tissues, namely the renal and pancreatic LNs (2). Kurts et al. have shown that these auto-reactive and *in vivo* activated OT-1 $CD8^+$ T cells are deleted in the draining LNs of OVA-expressing tissues (4). However, the adoptive transfer of a high number, i.e. $10x10^6$, of *in vitro* activated OT-1 $CD8^+$ T cells into RIP-mOVA mice leads to varying degrees of kidney infiltration, ranging from no infiltrates to dense mononuclear infiltration of the whole cortex. $CD8^+$ T cells have been found to accumulate next to the mOVA-expressing tubules (5). Beside the kidney infiltration, a destruction of pancreatic islet cells have appeared at day 2-3 after transfer, manifesting in diabetes. Importantly, the adoptive transfer of high numbers (up to $20x10^6$) of naïve as opposed to activated OT-1 $CD8^+$ T cells has not resulted in kidney infiltration whereas development of diabetes has always been observed even after the injection of low numbers of naïve OT-1 $CD8^+$ T cells (5).

Immunization with self antigens has turned out to be an efficient strategy to induce autoimmune diseases in mice. For example, in experimental autoimmune encephalomyelitis (EAE), the animal model for MS, the injection of myelin components, e.g. myelin basic protein (MBP) and proteolipid protein (PLP) in complete Freud's adjuvant has induced antigen-specific Th1 cells that infiltrate and accumulate in the central nervous system. There, T cells recognize their antigen and produce IFN-γ leading to inflammation and destruction of myelin which results in paralysis of the mice (6, 7). Thus, the injection of OVA-antigens into RIP-mOVA mice may be a promising strategy to induce an autoimmune response in the kidney.

The purpose of the present study has been to adapt this RIP-mOVA animal model to study
1) the characteristics of tubulointerstitial inflammation,
2) the role of the PD-1/PD-L1 pathway *in vivo* and
3) the development of strategies to influence autoimmune renal injury.

Two experimental strategies have been tested.

The first one was the adoptive transfer of *in vitro* activated OVA peptide-specific OT-1 $CD8^+$ T cells into RIP-mOVA mice (CTL-transfer strategy). According to the literature, after the transfer of high amounts of CTL, i.e. $10x10^6$ cells we expected that the OVA-specific OT-1 effector CTLs will find their targets, i.e. OVA-expressing renal tubular epithelial cells, and induce renal tubular inflammation and injury.

The second strategy was designed to immunize RIP-mOVA mice with OVA protein and peptide (immunization strategy). The aim was to induce an autoimmune response in RIP-mOVA mice against the kidney by injection of "self-antigens", e.g. $OVA_{257-264}$ peptide and OVA protein. Professional APCs such as DCs and macrophages will take up the injected antigens, process and present them to $CD4^+$ and $CD8^+$ T cells. This should lead to an activation and expansion of auto-reactive $CD4^+$ and $CD8^+$ T cells and to an induction of OVA-specific polyclonal immune responses *in vivo*. The *in vivo* primed OVA-specific $CD4^+$ and $CD8^+$ T cells should then infiltrate into the kidney and the pancreas.

4.3. Materials and methods

General reagents and antibodies

Cell culture reagents were purchased from Invitrogen (Gaithersburg, USA) and Sigma (St. Louis, USA). Anti-PD-L1 (αPD-L1) monoclonal antibody (Ab) for *in vivo* injection was obtained from hybridoma cell culture supernatants from the cell line MIH5 which was obtained from RIKEN Cell bank with the permission of Prof. Miyuki Azuma, Tokyo Medical and Dental University, Japan. Purification of the Ab from MIH5 cell culture supernatants was performed by Dr. David Hacker, Protein Expression Core Facility, EPFL-SV, Lausanne, Switzerland. The Abs were diluted in PBS. As isotype control, IgG from rat serum (Sigma-Aldrich, Germany) dissolved in PBS was used.

Purified rat anti-mouse MHC class II, CD4 (clone L3T4), CD8a (Ly-2, clone 53.6.7) and PD-L1 (clone MIH5) used for immunofluorescence were purchased from eBiocience (San Diego, USA). As secondary antibody for the immunofluorescence Cy™3-conjugated mouse anti-rat IgG- from Jackson ImmunoResearch (USA) was used. Anti-CD3 antibody (clone: CD3-12, rat anti-human, cross-reactive with mouse) employed for immunohistochemistry was obtained from Serotec (Oxford, UK).

Mice

RIP-mOVA and OT-1 mice were obtained from the Jackson Laboratory (Bar Harbor, Me., USA). C57BL/6 (B6) mice were purchased from the breeding facility of the University of Zürich (LTK Füllinsdorf, Basel, Switzerland). All mice were bred and maintained at the animal facility of the Institute of Physiology, University of Zürich-Irchel, Switzerland. The animal experimental protocols were approved by the Regulatory Commission for Animal Studies at the Canton of Zürich, Switzerland. RIP-mOVA (Tg/+), non-transgenic littermate control (non-Tg) and B6 mice were used at the age from 8-18 weeks for all *in vivo* experiments.

Screening for the presence of the transgene in RIP-mOVA and OT-1 mice

Genotyping of RIP-mOVA mice was performed by isolating DNA from a tail tip with the DNAeasy® Blood&Tissue kit (Qiagen, Hilden, Germany) according to the manufactures` protocol. Primers and PCR cycle conditions were taken from Kurts et al (2). Peripheral blood

of OT-1 mice was screened for OVA-peptide specific $CD8^+$ T cells by FACS analysis as described below.

In vitro activation and adoptive transfer of OT-1 $CD8^+$ T cells

$CD8^+$ T cells were isolated from spleens of naïve OT-1 mice by positive selection with anti-mouse CD8a (Ly-2) MACS microbeads obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). $1x10^6$ OT-1 $CD8^+$ T cells were activated *in vitro* by stimulation with $4x10^6$ $CD8^-$ splenocytes pulsed with 1 µg/ml of chicken ovalbumin peptide (OVA 257–264, amino acid sequence SIINFEKL, Proimmune, London, UK). Cells were incubated in IMDM medium supplemented with 5 µM 2-mercaptoethanol, 0.05mg/ml gentamycin and 10% FBS. After 3-5 days of stimulation activated OT-1 $CD8^+$ T cells were injected intravenously (i.v.) into RIP-mOVA, non-transgenic littermate controls or B6 mice.

Treatment with anti-PD-L1 antibody (αPD-L1) *in vivo*

αPD-L1 was injected intraperitonially (i.p.) into RIP-mOVA and B6 mice on day 1 and 3 after adoptive transfer of $2.5x10^6$ or $10x10^6$ OT-1 $CD8^+$ T cells. 200 µg of Ab was injected each time. As controls, groups of RIP-mOVA and B6 mice were treated with PBS or isotype control (200µg, rat IgG) at the same time after adoptive transfer.

Immunization protocol

RIP-mOVA (Tg/+), non-transgenic controls (non-Tg) and B6 mice were immunized subcutaneously in the base of the tail with 100 µg albumin from chicken egg white (Sigma-Aldrich, St. Louis, USA) and 50 µg $OVA_{257–264}$ emulsioned in the incomplete Freud's adjuvant (IFA, Sigma-Aldrich, St. Louis, USA). Mice were either immunized once (day 0), twice (day 0 and 7) or three times (day 0, 7 and 14), respectively. The mice were sacrificed on different time points after immunization, e.g. on day 6, 14, 20 and 30.

Measurement of glucose in urine and blood

The disease progression, specifically the onset of diabetes, was determined by daily measurement of glucose levels in the urine and/or the blood. Combur^{3}Test® or Combur^{10}Test® M (Roche, Basel, Switzerland) were used to analyze glucosuria. Glucose levels between 2.8-55 mmol/l could be detected by these urine strips. Blood glucose levels were determined with Ascensia® CONTOUR® (Bayer Schweiz AG, Zürich, Switzerland) and were measured twice per day in experimental mice after CTL transfer.

Insulin treatment

RIP-mOVA mice injected with OT-1 $CD8^+$ T cells were treated with insulin (Actrapid® or Levemir® Penfill® 3, both novo nordisk®) when glycemia was higher than 8 mmol/l. Mice received 0.5 U/kg Actrapid® twice per day or Levemir® Penfill® 3 once per day.

Organ removal

At the end of the experiments mice were sacrificed by cervical dislocation and organs were removed for further analysis. The spleen was harvested for FACS analysis and *in vitro* experiments. Each kidney was divided into 2 pieces. 2 kidney pieces were snap frozen in isopentan (Fluka, Germany) in liquid nitrogen and 1 piece was fixed in formalin. All three were used for histological examination either on cryopreserved or formalin fixed tissue. The last one was transferred into RNA later® (Ambion, USA) for RNA isolation. The pancreas was either fixed in formalin for analysis of histology or stored in RNA later.

Analysis of histology

Haematoxylin and eosin (H&E), Period acid-Schiff (PAS) and CD3 staining were performed on formalin-fixed, paraffin-embedded kidney samples and done by Stephan Segerer. Staining of CD3 positive cells was performed as described (8). The tubulointerstitial T cell infiltrate was assessed by counting CD3 positive cells in 15 high power fields (HPFs) and by calculating the mean CD3 infiltrating cells per HPF.

Cryo-preserved kidneys were cut into 6 µm sections (microtome, Leica CM3050 S) and placed on Superfrost® Plus slides. The slides were fixed in ice-cold acetone for 10 min and then washed in PBS. The following incubations steps were performed in a wet chamber. The slides were blocked for 1 h in 5% goat serum diluted in PBS and then incubated overnight at 4°C with primary antibodies against mouse MHC class II, PD-L1, CD8 and CD4 diluted in PBS containing 1% BSA. Next day, the slides were washed in PBS for three times and stained with the secondary Ab and DAPI (4',6-Diamidin-2'-phenylindoldihydrochlorid) for 1 h at RT in the dark. After washing, slides were covered with ProLong® Gold (Invitrogen, USA) and analyzed by microscope (Zeiss Axiovert 200M, Germany).

FACS analysis

OVA-peptide specific $CD8^+$ T cell population in experimental mice after CTL transfer was measured in splenocytes with the Pro5 MHC Pentamer-PE against SIINFEKL (Proimmune,

London, UK) according to the manufacture's protocol. $CD8^+$ T cells were stained with CD8-FITC (eBioscience, San Diego, USA).

Measurement of OVA-antigen specific T cell responses

Antigen-specific $CD8^+$ T cell response:

$CD8^+$ T cells were isolated from spleens and LNs from immunized RIP-mOVA, non-transgenic littermates and B6 mice with anti-mouse CD8a (Ly-2) MACS microbeads as described above. Part of the CD8 negative cell fraction was used as APCs and was pulsed with 1 µg/ml $OVA_{257–264}$ for 1h at 37°C. $2x10^6$ $CD8^+$ T cells were then stimulated with $3x10^6$ $CD8^-$ cells loaded with or without $OVA_{257–264}$ in 24-well plates. After 48 h co-culture the supernatant was collected to measure the IFN-γ production by re-stimulated $CD8^+$ T cells using mouse IFN-γ specific ELISA (BD OptEIA™, BD Biosciences, San Diego, USA).

Antigen-specific $CD4^+$ T cell response:

The other part of the $CD8^-$ cells from spleen and LNs were used to determine the OVA-specific $CD4^+$ T cells response. $5x10^5$ $CD8^-$ negative cells were stimulated with OVA protein at concentrations of 100, 20, 4, 0.8, and 0.16 µg/ml for 72 h in triplicates in 96-well plates. As a positive control for $CD4^+$ T cell proliferation, cells were stimulated with 2 µg/ml ConA at the same time. The $CD4^+$ T cell proliferation was measured with the non-radioactive colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol. Results were presented as the mean value of the OD at 490 nm.

Statistical analysis

All *in vitro* experiments were performed as duplicate or triplicate. The preparation of the graphs and the statistical analysis was performed with GraphPad Prism Version 4 (GraphPad Software, Inc; La Jolla, USA). Mann Whitney test was used to compare the different groups of mice. A significant difference in the onset of diabetes development was tested with the Logranktest. Significance was accepted at $p \leq 0.05$.

4.4. Results

4.4.1. CTL transfer strategy

Adoptive transfer of 10x10^6 activated OT-1 CTLs into RIP-mOVA mice induced antigen-specific pancreas and renal inflammation

Diabetes development in RIP-mOVA mice

The adoptive transfer of high amounts of activated OT-1 CD8$^+$ CTLs into RIP-mOVA mice should lead to pancreas and kidney infiltration (5). After i.v. injection of 10x10^6 of *in vitro* activated effector OT-1 CTLs into RIP-mOVA (Tg/+), non-transgenic littermate control (non-Tg) or B6 mice, 60% of the RIP-mOVA mice developed glucosuria on day 3. At day 5, 80% of the RIP-mOVA mice showed high levels of glucosuria (55 mmol/l). Non-transgenic littermate controls or B6 mice did not develop glucosuria until the end of the experiment on day 5 (Fig. 2).

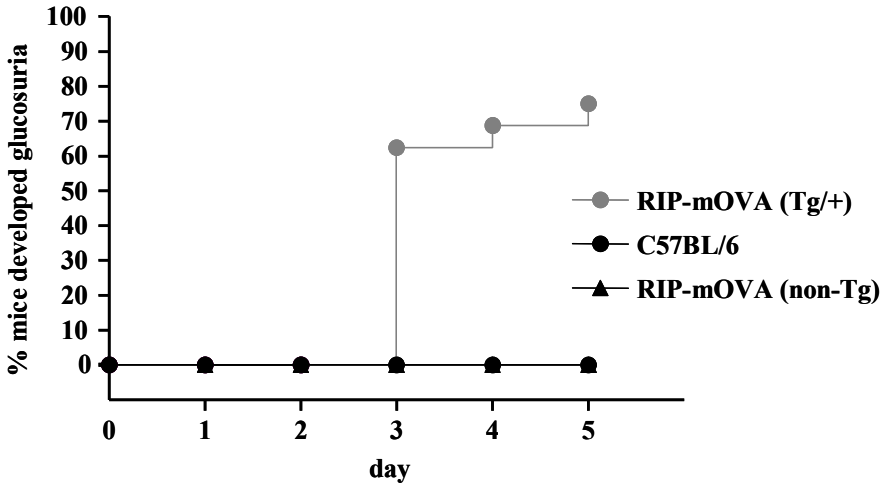

Figure 2: The onset of glucosuria in RIP-mOVA mice, non-transgenic littermates and C57BL/6 mice after adoptive transfer of 10x10^6 activated OT-1 CD8$^+$ T cells. The figure depicts a summarization of all the experiments in which mice were transferred with 10x10^6 activated OT-1 CD8$^+$ T cells. RIP-mOVA (Tg/+) mice (n=16); non-transgenic (non-Tg) littermates (n=9); C57BL/6 mice (n=14).

The overview staining with H&E on the pancreas sections of glucosuric RIP-mOVA mice revealed a complete destruction of the architecture compared to the control animals (Fig. 3).

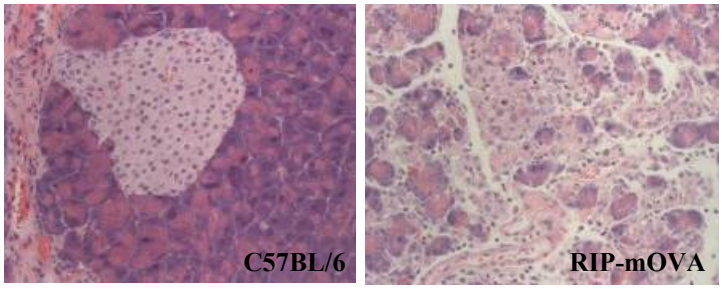

Figure 3: H&E staining of pancreas sections of C57BL/6 and RIP-mOVA mice 5 days after adoptive transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells. Representative for n=3 animals/group.

Induction of kidney inflammation and infiltration in RIP-mOVA mice

Then the assessment of kidney infiltration and inflammation after the CTL transfer was performed on sections of cryopreserved kidneys. Focal accumulation of $CD8^+$ positive T cells was detected around many tubular epithelial cells (Fig. 4B). The kidney sections of control B6 mice showed only few positive $CD8^+$ T cells and no cluster of these cells around the tubules was observed (Fig. 4A), suggesting that the positively stained $CD8^+$ T cells in the kidneys of RIP-mOVA mice were the transferred OT-1 $CD8^+$ T cells. There was a slight increase in the number of $CD4^+$ T cells in the kidneys of RIP-mOVA mice compared to the B6 controls (Fig. 4C and D). Interestingly, MHC class II expression was strongly induced on TECs in RIP-mOVA mice (Fig. 4F) but not on tubules of B6 mice (Fig. 4E). MHC class II positive interstitial cells, most likely DC (9), were found in both groups. Similarly, strong staining of PD-L1 was detected on the tubular cells of RIP-mOVA mice (Fig. 4H) but not on the tubules of control B6 mice (Fig. 4G).

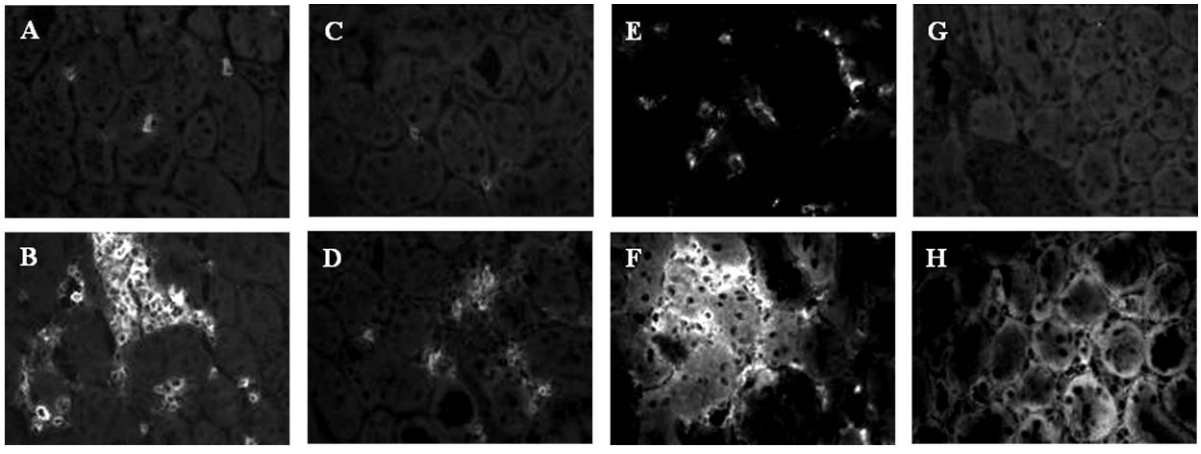

Figure 4: Immunofluorescence staining of kidney sections from C57BL/6 mice (A, C, E, G) and RIP-mOVA mice (B, D, F, H) 5 days after injection of $10x10^6$ activated OT-1 $CD8^+$ T cells. Sections of cryopreserved kidneys were stained with anti-mouse antibodies against (A, B) $CD8^+$, (C, D) $CD4^+$, (E, F) MHC class II and (G, H) PD-L1.Magnification 40x.

Taken together, the adoptive transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells into RIP-mOVA mice induced a focal accumulation of $CD8^+$ T cells around tubules accompanied by an induction of MHC class II and PD-L1 expression on tubular epithelial cells.

To test the hypothesis that PD-L1 on parenchymal tissue, (e.g. the kidney and the pancreas) has a protective role, the next experiments were aimed to inhibit the renal epithelial PD-1/PD-L1 pathway *in vivo*. This was performed by administration of specific anti- PD-L1 monoclonal Ab (αPD-L1) or PBS after transferring $10x10^6$ OT-1 CTLs into RIP-mOVA or B6 mice, respectively. Both αPD-L1 or PBS treatment groups developed glucosuria on day 3. At the end of the experiment (day 5), 80% of the RIP-mOVA mice treated with αPD-L1, and 60% of the RIP-mOVA mice injected with PBS had displayed glucosuria (difference n.s., Fig. 5).

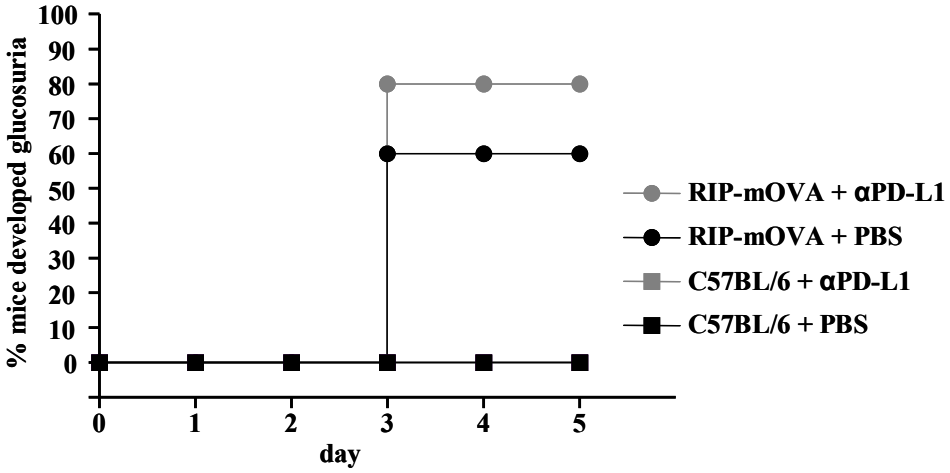

Figure 5: Development of glucosuria in RIP-mOVA and C57BL/6 mice treated either with αPD-L1 or PBS after adoptive transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells. RIP-mOVA mice + αPD-L1 (n=5); RIP-mOVA mice + PBS (n=5); C57BL/6 mice + αPD-L1 (n=3); C57BL/6 mice + PBS (n=3).

The measurement of blood glucose was started in the afternoon of day 3 when the onset of glucosuria had been detected. All of the RIP-mOVA mice, except one treated with αPD-L1, exhibited elevated blood glucose levels (> 8 mmol/l) in the afternoon at day 3. All of the RIP-mOVA mice were treated with insulin Actrapid®. Since this drug has only a half life of 3-4 h, the blood glucose levels were raised the next morning and the mice were treated with insulin twice on day 4 when blood glucose levels were > 8 mmol/l. There was no significant difference in the levels of blood glucose between both RIP-mOVA groups over the experimental period after the onset of glycaemia (Fig. 6). B6 mice treated either with αPD-L1

or PBS after the CTL transfer did not develop glucosuria and glycemia until the end of the experiment (Fig.5 and 6).

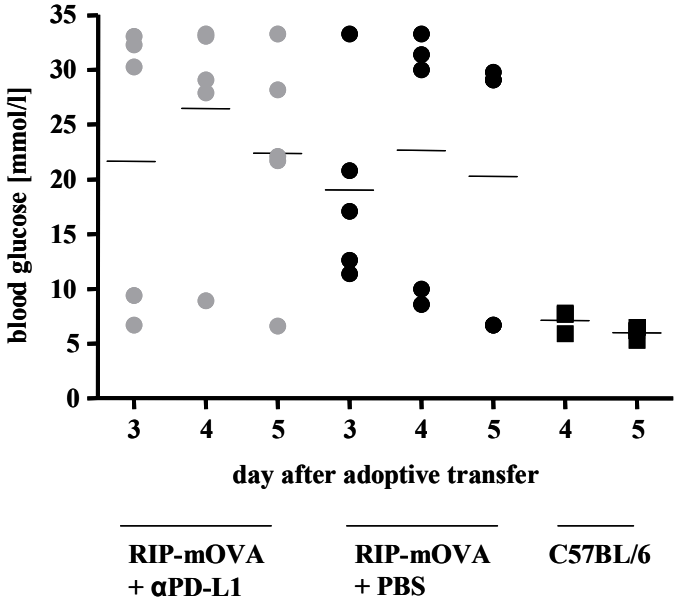

Figure 6: Blood glucose levels on day 3-5 after adoptive transfer of $10x10^6$ OT-1 $CD8^+$ T cells. The values on day 4 represent the mean of the morning and the afternoon measurements whereas the levels on day 3 were determined in the afternoon and on day 5 in the morning before termination of the experiment. The blood glucose in C57BL/6 mice was measured only on day 4 and 5. RIP-mOVA mice + αPD-L1 (n=5); RIP-mOVA mice + PBS (n=5); C57BL/6 mice (n=3).

The tubulointerstitial infiltration in the 4 different groups was assessed by overview staining with PAS on sections from formalin-fixed kidneys. Tubulointerstitial infiltration and focal accumulation of cells around tubules was detected in kidneys of both groups of RIP-mOVA mice with or without αPD-L1 treatment (Fig. 7C and D). However, the PAS staining revealed no obvious difference in the level/rate of infiltration between RIP-mOVA mice treated with αPD-L1 or PBS. The kidneys of B6 mice were not infiltrated (Fig. 7A and B).

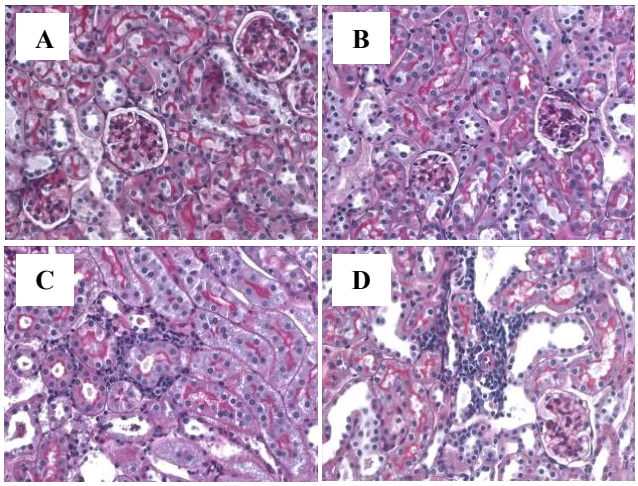

Figure 7: Representative pictures of the PAS staining of sections from formalin-fixed kidneys at day 5 after transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells into RIP-mOVA and C57BL/6 mice. (A) C57BL/6 + αPD-L1 (n=3); (B) C57BL/6 mice + PBS (n=3); (C) RIP-mOVA mice + αPD-L1 (n=5); RIP-mOVA mice + PBS (n=5). Magnification 25x.

CD3 staining (as a marker for T cells) was performed to better quantify the infiltration. The kidneys of RIP-mOVA mice that were treated with αPD-L1 showed a significant higher number of infiltrated $CD3^+$ cells compared to the kidneys of B6 mice (Fig. 8) but different levels of infiltration were not detectable between kidneys of RIP-mOVA mice either treated with αPD-L1 or PBS. One mouse of the RIP-mOVA mice treated with αPD-L1 and two mice of the RIP-mOVA mice injected with PBS had low or no infiltration of $CD3^+$ cells in the kidneys after adoptive transfer. These were the same mice that had not developed glycemia (Fig. 8).

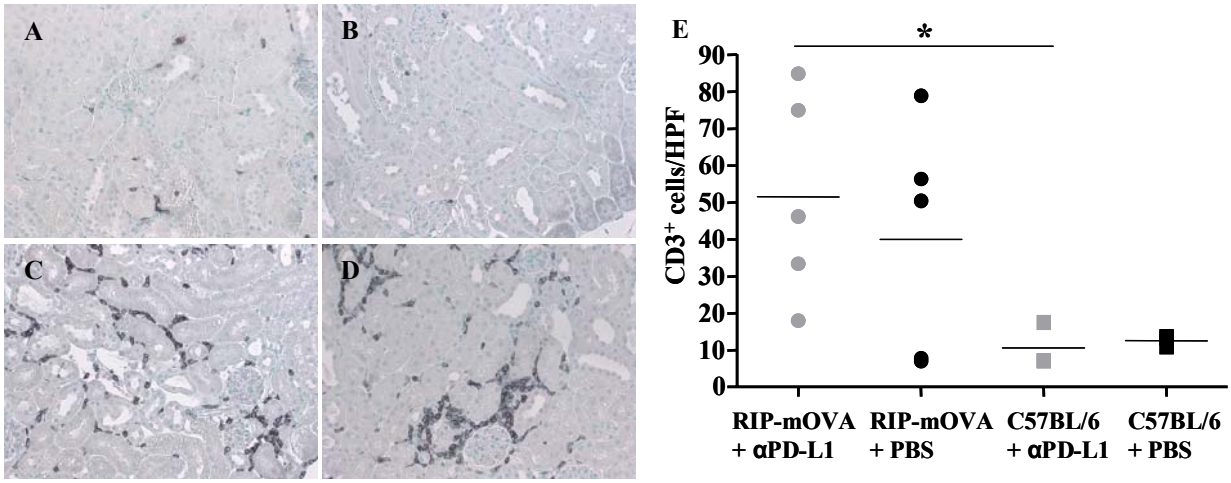

Figure 8: (A-D) Representative pictures of the CD3 staining of sections from formalin-fixed kidneys at day 5 after transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells into RIP-mOVA and C57BL/6 mice. (E) Quantification of the CD3 staining. (A) C57BL/6 + αPD-L1 (n=3); (B) C57BL/6 mice + PBS (n=3); (C) RIP-mOVA mice + αPD-L1 (n=5); RIP-mOVA mice + PBS (n=5). Magnification 25x. (E) * < 0.05 analyzed with Mann-Whitney U-test.

In summary, treatment of RIP-mOVA mice with αPD-L1 antibody or PBS after adoptive transfer of $10x10^6$ OT-1 $CD8^+$ T cells revealed no significant differences in the onset/severity of diabetes and the level of tubulointerstitial infiltration.

Retrieval of OVA-specific $CD8^+$ T cells in RIP-mOVA and B6 mice 5 days after transfer

At day 5 after adoptive transfer, the presence of OT-1 $CD8^+$ T cells in splenocytes of RIP-mOVA or B6 mice was determined using pentamer staining and was compared with splenocytes of OT-1 mice. As a positive control, 95% of the $CD8^+$ T cells of OT-1 mice were specific for $OVA_{257-264}$ peptide (Fig. 9A). OVA-peptide specific $CD8^+$ T cells could not be detected in naïve RIP-mOVA and B6 mice (data not shown). B6 mice had still 27-51%

specific $CD8^+$ T cells in the spleen after adoptive transfer (Fig. 9C and D). In contrast, RIP-mOVA mice, regardless of their treatment, exhibited a significant lower population of $OVA_{257-264}$ peptide $CD8^+$ T cells (7-16%). There was no significant difference in the OVA-peptide specific $CD8^+$ T cell population between the RIP-mOVA mice treated with αPD-L1 or PBS (Fig. 9D). The significant lower number of specific $CD8^+$ T cells in the spleen of RIP-mOVA compared to the B6 mice suggested that the transferred $CD8^+$ T cells might home to the OVA-expressing tissues namely the pancreas and the kidney.

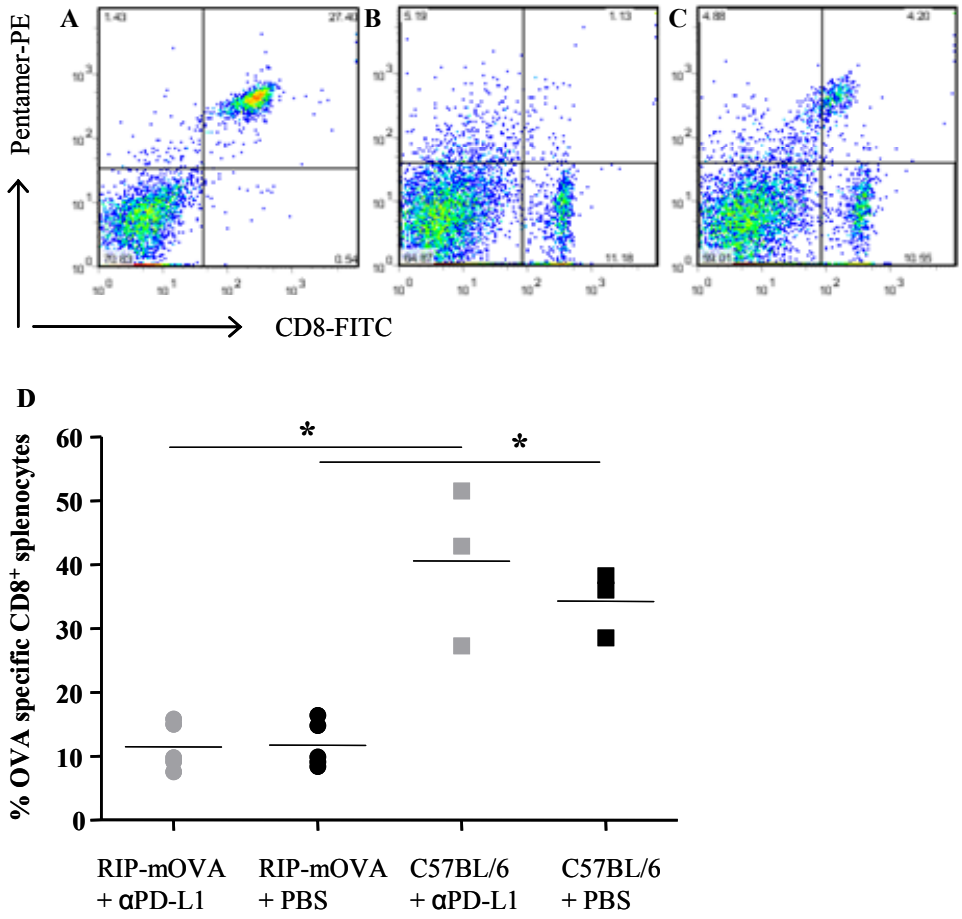

Figure 9: FACS staining of $OVA_{257-264}$ peptide-specific $CD8^+$ T cells in the spleens of RIP-mOVA and C57BL/6 mice at day 5 after transfer of $10x10^6$ activated OT-1 $CD8^+$ T cells. $1x10^6$ splenocytes were stained with anti-mouse CD8-FITC and Pro5 MHC Pentamer-PE against SIINFEKL to test the specificity of $CD8^+$ T cells. Representative FACS blots of the staining of splenocytes from (A) naïve OT-1 mice as a positive control; (B) RIP-mOVA mice after adoptive transfer and (C) C57BL/6 mice after adoptive transfer. (D) Comparison of the relative number of OVA-specific $CD8^+$ T cells between RIP-mOVA and C57BL/6 mice either treated with αPD-L1 or PBS. RIP-mOVA mice + αPD-L1 (n=5); RIP-mOVA mice + PBS (n=5); C57BL/6 mice + αPD-L1 (n=3); C57BL/6 mice + PBS (n=3). * < 0.05 analyzed with Mann-Whitney U-test.

In addition, these results suggested that the injected cells could still be found in the circulation of these mice and had not been depleted on day 5 after adoptive transfer.

Adoptive transfer of a lower amount ($2.5x10^6$) of activated OT-1 $CD8^+$ T cells

It might be that the number of $10x10^6$ of activated OT-1 CTL was too high to distinguish differences in the onset of diabetes and kidney infiltration in RIP-mOVA mice after the administration of αPD-L1. Consequently, a lower number of activated OT-1 $CD8^+$ T cells was transferred.

After the transfer of $2.5x10^6$ activated OT-1 CTLs RIP-mOVA mice were also treated with αPD-L1 or isotype control (rat IgG) on day 1 and 3. Insulin Levemir® Penfill® 3 was injected into the mice when blood glucose levels were > 8 mmol/l.

As shown in Figure 10, 40% of the RIP-mOVA mice treated with αPD-L1 developed diabetes on day 3 after adoptive transfer of $2.5x10^6$ activated OT-1 $CD8^+$ T cells. At day 5, all of the mice of this group displayed glycemia (Fig. 10A). 30% of the RIP-mOVA mice injected with the isotype control developed diabetes on day 4 after the CTL transfer and all of the mice were glycemic on day 6 when the experiment was terminated. Although, the levels of blood glucose measured on day 3 and 4 in RIP-mOVA mice with αPD-L1 treatment seemed to be higher than in the RIP-mOVA isotype group, they did not differ statistically and this difference was diminished after day 5 when almost all mice in both groups became diabetic.

Tubulointerstitial infiltration was hardly detectable after adoptive transfer of $2.5x10^6$ activated OT-1 $CD8^+$ T cells. Only a few cells in the interstitium could be found. There was no obvious difference in the level of infiltration between RIP-mOVA mice treated with αPD-L1 or PBS (data not shown).

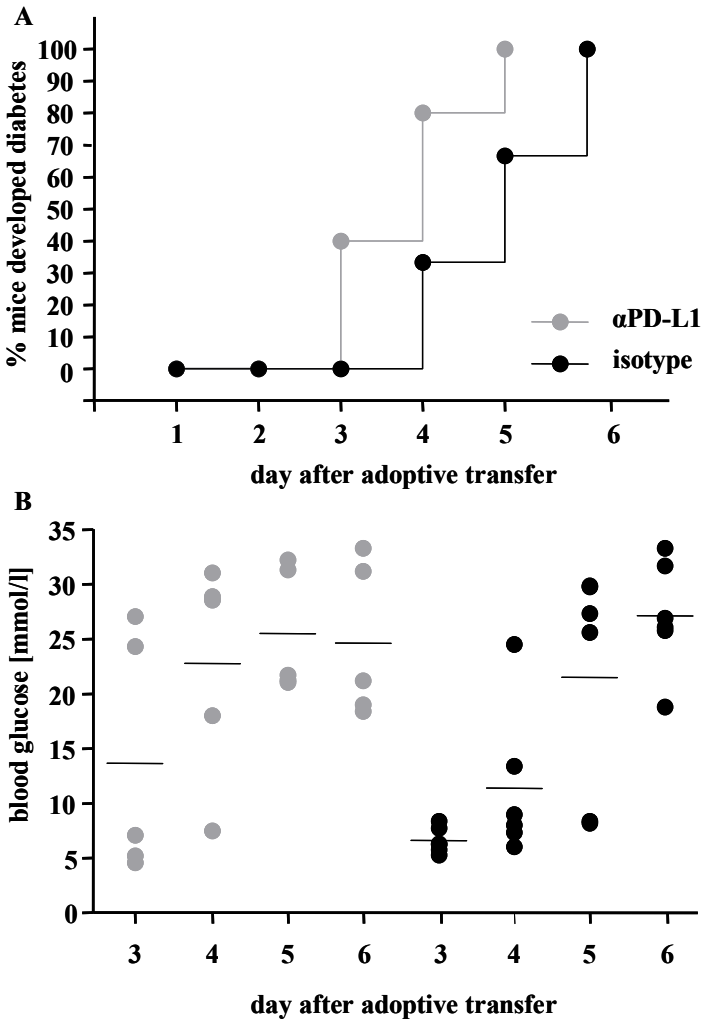

Figure 10: Diabetes development in RIP-mOVA mice treated either with αPD-L1 or isotype after transfer of 2.5x10^6 activated OT-1 CD8$^+$ T cells. (A) Onset of glycemia, p=0.0529 (n.s.) (B) Blood glucose levels on day 3 to 6 after adoptive transfer. The values on day 4 and 5 represent the mean of the morning and the afternoon measurements whereas the levels on day 3 were determined in the afternoon and on day 6 in the morning before termination of the experiment. RIP-mOVA + αPD-L1 (n=5), RIP-mOVA mice + isotype (n=6).

4.4.2. Immunization strategy

Summary of the immunization strategies

Transgenic and non-transgenic RIP-mOVA were immunized with 100 µg OVA protein and 50 µg $OVA_{257-264}$ peptide in incomplete Freud's adjuvant. Different protocols regarding the amount of immunizations and the duration of the experiment were tested. A summary of the performed immunization experiments including the number of immunizations, animals and the day of termination of the experiment are depicted in Table 1.

Table 1: Summary of the immunization experiments

RIP-mOVA	n x immunization	day after 1st immunization (d)	animal number (n)	tubular inflammation (n)
non-Tg	1x	6	4	0/4
non-Tg	2x	14	5	0/5
non-Tg	2x	20	3	0/3
non-Tg	3x	30	3	0/3
Tg/+	1x	6	5	2/5
Tg/+	2x	14	4	4/4
Tg/+	2x	20	2	2/2
Tg/+	3x	30	3	3/3

non-transgenic (non-Tg), transgenic (Tg/+). Tubular inflammation was determined by the observation of MHC class II induction on TECs.

One immunization with OVA antigens induced tubular inflammation which was determined by MHC class upregulation on TECs (10, 11) in two out of five RIP-mOVA mice (Tg/+). Consequently, mice were repeatedly immunized in the following experiments and different time points after the second immunization were investigated. All of the immunized RIP-mOVA mice showed upregulation of MHC class II expression on TECs after the second and third immunization. None of the non-transgenic mice or B6 mice (data not shown) developed tubular inflammation.

Development of glucosuria

In total only 4 animals of all immunized RIP-mOVA mice developed glucosuria (levels were between 5.5-17mmol/l). Glucosuria was observed in two animals after the second immunization on day 14 and in two mice on day 20. Mice that were immunized thrice did not develop glucosuria (data not shown).

Induction of kidney inflammation

A mild infiltration of $CD4^+$ and $CD8^+$ T cell into the kidneys of RIP-mOVA mice was observed after two (Fig. 11B, D) and three times of immunization. The T cells were overall distributed and accumulation of T cells around tubules was only found sporadically. Non-transgenic controls or B6 mice only showed single cells being positive for CD4 and CD8 staining (Fig. 11A and C). MHC class II expression was strongly induced on many TECs in RIP-mOVA mice after immunization (Fig. 11F) whereas tubules of non-transgenic or B6 mice only showed interstitial MHC class II expression (Fig. 11E). Tubules as well as glomeruli and vessels showed a strong staining of PD-L1 (Fig. 11H) in RIP-mOVA mice whereas kidneys of non-transgenic and B6 mice were negative for the expression of PD-L1 (Fig. 11G).

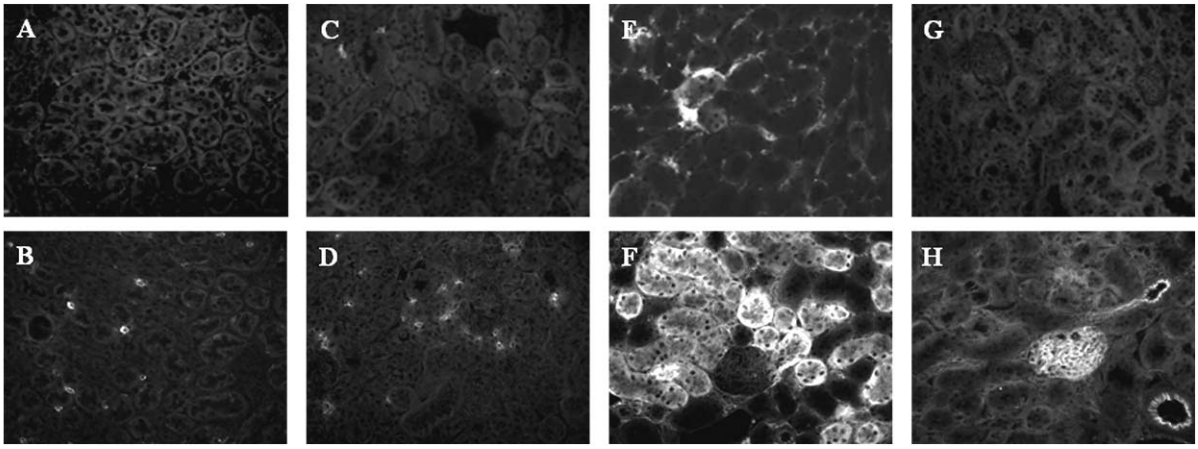

Figure 11: Immunofluorescence staining of kidney sections from non-transgenic RIP-mOVA/B6 mice (A, C, E, G) and RIP-mOVA mice (Tg/+) (B, D, F, H) which were immunized twice, day 14. The pictures are as well representative for mice immunized twice, day 20 and thrice, day 30. Sections of cryopreserved kidneys were stained with anti-mouse antibodies against (A, B) $CD8^+$, (C, D) $CD4^+$, (E, F) MHC class II and (G, H) PD-L1. Magnification 40x.

Immunization with "self-antigens" did not induce tolerance in RIP-mOVA mice

In vivo development of OVA-specific T cells after immunization was determined by *ex vivo* stimulation of $CD4^+$ and $CD8^+$ T cells with OVA protein and $OVA_{257\text{-}264}$ peptide, respectively. $CD8^+$ T cells isolated from immunized transgenic, non-transgenic RIP-mOVA and B6 mice produced IFN-γ after exposure with splenocytes loaded with $OVA_{257\text{-}264}$ peptide whereas $CD8^+$ T cells pulsed with splenocytes not loaded with peptide did not secrete IFN-γ (Fig. 12A). An OVA-protein specific proliferation of $CD4^+$ T cells derived from immunized mice was as well observed (Fig. 12B). T cells isolated from non-immunized (naïve) RIP-mOVA mice did not show antigen-specific T cell responses (data not shown).

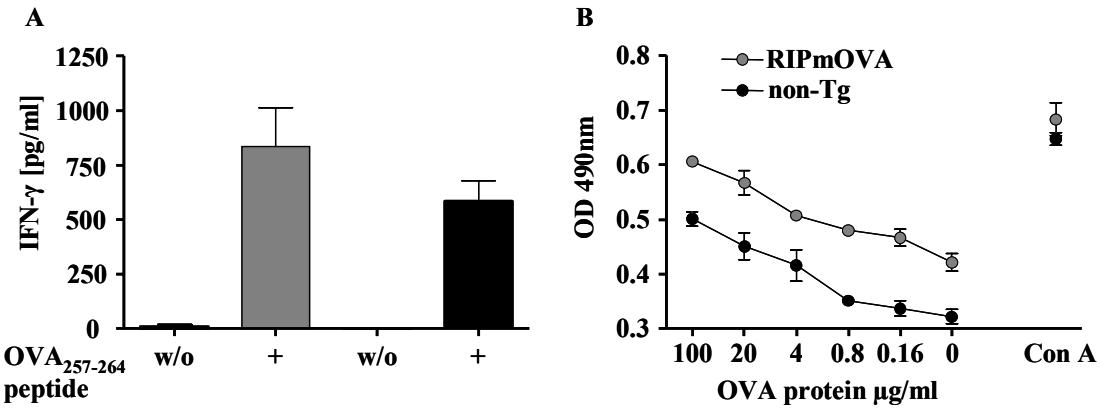

Figure 12: Representative graphs of (A) IFN-γ production of OVA-peptide specific CD8$^+$ T cells and (B) OVA-protein specific CD4$^+$ T cell proliferation. T cells were isolated from spleens and LNs of immunized RIP-mOVA and non-transgenic controls. (A) CD8$^+$ T cells were stimulated with splenocytes loaded with (+) or without (w/o) $OVA_{257-264}$ peptide for 48 h. (B) CD4$^+$ T cells were stimulated with different concentrations of OVA-protein as indicated for 72 h. ConA was used as a positive control for CD4$^+$ T cell proliferation. The graph shows the T cell responses of one animal/group after two times immunization, day 14 but the pattern and the antigen-dependent response are representative for transgenic, non-transgenic RIP-mOVA and B6 mice which were immunized once, twice and thrice.

One immunization with OVA-antigens was efficient to generate antigen-specific T cells in transgenic and non-transgenic RIP-mOVA and B6 mice (data not shown). The antigen-dependent *ex vivo* T cell response of immunized RIP-mOVA mice indicated that these RIP-mOVA mice, although they express OVA-antigens as self antigens, developed antigen-specific/self-reactive T cells *in vivo* and that tolerance against self-antigens was not induced in these mice.

4.5. Discussion

Two different approaches were used to investigate the expression and the role of renal epithelial PD-L1 *in vivo*. The first one was to use the established (5) protocol for adoptive transfer of activated OT-1 $CD8^+$ T cells into RIP-mOVA mice and the second one was to immunize RIP-mOVA mice with self-antigens to induce a pathophysiological autoimmune response in the kidney.

RIP-mOVA mice developed diabetes after the transfer of activated OT-1 $CD8^+$ T cells, indicating that the method published by Kurts et al. is reproducible and reliable (5). The incidence of 70-100% diabetes development in RIP-mOVA mice after the transfer of high and low amounts of OT-1 $CD8^+$ T cells suggested that some of the animals might have a different expression of mOVA. Another possibility might be that the termination of the experiment on day 5 after the transfer of high amounts was too early. Mice that did not suffer from diabetes also did not demonstrate kidney infiltration. Consequently, only mice developing diabetes should take into account for the investigation of kidney pathology/inflammation after transfer of OT-1 $CD8^+$ T cells in the future experiments.

The transfer of high amounts of self-reactive OT-1 $CD8^+$ T cells into RIP-mOVA mice caused a rapid accumulation of $CD8^+$ T cells around kidney tubules certainly OVA-expressing TECs as described (5). Furthermore, TECs surrounded by $CD8^+$ T cells showed an induction of MHC class II and PD-L1 expression suggesting a local inflammatory environment. For instance, IFN-γ secretion by infiltrating $CD8^+$ T cells could be responsible for the induction of MHC class II and PD-L1 on TECs. This needs to be confirmed in future experiments investigating the intrarenal expression of cytokines, e.g. IFN-γ or TNF-α that are known to upregulate these molecules on TECs. The immunization of RIP-mOVA mice with "self"-antigens, i.e. OVA peptide and protein, caused only mild T cell infiltration into the kidneys but induced as well a marked upregulation of MHC class II and PD-L1. Interestingly, PD-L1 expression was not only found on TECs but as well on glomeruli and vessels of the kidney. One might speculate that the immunization caused a systemic inflammatory cytokine response leading to a broad upregulation of MHC class II and PD-L1 in kidneys in contrast to the CTL transfer.

Although OVA is a self-antigen of RIP-mOVA mice, the immunization with OVA peptide and protein primed OVA-specific $CD4^+$ and $CD8^+$ T cells *in vivo*, as assessed by *ex vivo*

antigen-specific T cell responses. Consequently, RIP-mOVA mice were not tolerant and developed auto-reactive T cells after immunization with “self”-antigens. Nevertheless, only four mice had glucosuria, suggesting infiltration and destruction of the pancreas by *in vivo* primed auto-reactive T cells. The same animals exhibited strong MHC class II and PD-L1 expression in the kidney. However, even repeated immunizations with OVA-antigens were not powerful enough to induce moderate T cell infiltration into kidneys and probably the pancreas as well.

The induction of PD-L1 on TECs in RIP-mOVA mice after the transfer of OVA-specific CTL and after the immunization with OVA antigens suggested an involvement of the PD-1/PD-L1 pathway in autoimmune pancreas and kidney responses. However, the blockade of this pathway by monoclonal antibodies against PD-L1 on day one and three after the transfer of high amounts of activated OT-1 CTL did not change the disease development such as the onset of diabetes and kidney infiltration. This might be due to the fact that the number of $10x10^6$ activated $CD8^+$ T cells was too high to distinguish differences in the outcome. Consequently, a lower amount of activated OT-1 $CD8^+$ T cells ($2x10^6$) was transferred. Although the transfer of lower numbers of CTLs did efficiently induce diabetes, no significant kidney infiltration was detected which is in agreement with the literature (5).

Blockade of PD-L1 with the same clone of Ab caused diabetes in RIP-mOVA mice after transfer of $5x10^5$ naïve OT-1 CTL whereas this low amount of OVA-specific $CD8^+$ T cells alone was not sufficient to induce diabetes (12). The inhibition of PD-L1 after the transfer of the lower amount of activated OT-1 CTL did not change the onset of diabetes significantly, but a tendency towards an earlier onset was seen in RIP-mOVA mice treated with αPD-L1. This suggests that the number of $2.5x10^6$ CTL was still too high to observe differences in the diabetes outcome. However, this amount was too low to induce tubulointerstitial infiltration in our experimental setup, and the treatment with αPD-L1 was not sufficient to trigger kidney infiltration. This result leaves open questions whether the injected antibody was able to reach the kidney and to block renal tubular epithelial expression. In addition, the focal PD-L1 expression on certain tubules induced by the CTL transfer method may not have been strong enough to alter the total disease development and therefore inhibition of PD-L1 by αPD-L1 mAbs was not sufficient to enhance kidney infiltration.

Another possibility to interrupt the PD-1/PD-L1 pathway *in vivo* is to transfer activated OT-1 CTLs that lack the receptor PD-1. RIP-mOVA mice developed diabetes after transfer of PD-1$^{-/-}$ OT-1 CTLs whereas transfer of wt OT-1 CTLs was not sufficient to induce diabetes (13). This approach might work as well to enhance kidney infiltration and could be one of the future experiments.

Anti PD-L1 treatment in the immunized RIP-mOVA mice might be a more suitable approach, since 1) the immunization protocol induced a broad expression of PD-L1 in kidneys and this might be more powerful to modulate T cell infiltration. Anti-PD-L1 Ab administration will clarify whether high expression of PD-L1 in kidneys limits infiltration of T cells and is protective, or if there is the necessity to develop a stronger immunization protocol to break the tolerance. 2). The first immunization experiments indicate that there is less influence on the occurrence of diabetes.

Nevertheless, a recent study has shown that the PD-1/PD-L pathway limited autoimmune-mediated kidney diseases. The expression of PD-L1, PD-L2 and PD-1 was increased in kidneys during nephrotoxic serum nephritis (NSN). In mice lacking PD-L1 (PD-L1$^{-/-}$), PD-L2 (PD-L2$^{-/-}$) or both molecules (PD-L1/L2$^{-/-}$) kidney pathology, loss of renal function and intrarenal leukocyte infiltration were enhanced compared to wild type mice during NSN. Specifically, activated CD8^{+} T cells were increased in diseased kidneys of PD-L1$^{-/-}$ mice whereas the absence of PD-L2 enhanced infiltration of macrophages. However, this study has demonstrated that PD-L1 expression on kidney parenchymal cells was not responsible for limiting leukocyte infiltration but rather PD-L1 on hematopoietic cells (14).

In conclusion, the transfer of high amounts of activated OVA-specific CD8^{+} CTL and the immunization with OVA antigens represent two different strategies to induce kidney inflammation and mild to moderate kidney infiltration in RIP-mOVA mice. Both experimental approaches are useful tools to investigate the expression of molecules including PD-L1 in autoimmune kidneys. The CTL transfer protocol is suitable to study acute tubulointerstitial nephritis whereas the immunization strategy may be used to investigate chronic autoimmune tubulointerstitial inflammation and infiltration. However, the pancreas seems to be more prone to autoimmune responses in this model than the kidney. Therefore, tubulointerstitial nephritis will always be accompanied by severe islet cell injury and the development of type 1 diabetes.

4.6. References

1. Schoop, R., P. Wahl, M. Le Hir, U. Heemann, M. Wang, and R. P. Wüthrich. 2004. Suppressed T-cell activation by IFN-γ-induced expression of PD-L1 on renal tubular epithelial cells. *Nephrol Dial Transplant* 19: 2713-2720.
2. Kurts, C., W. R. Heath, F. R. Carbone, J. Allison, J. F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* 184: 923-930.
3. Clarke, S. R., M. Barnden, C. Kurts, F. R. Carbone, J. F. Miller, and W. R. Heath. 2000. Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunol Cell Biol* 78: 110-117.
4. Kurts, C., H. Kosaka, F. R. Carbone, J. F. A. P. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive $CD8^+$ T Cells. *J Exp Med* 186: 239-245.
5. Kurts, C., I. Klebba, G. M. Davey, K. M. Koch, J. F. A. P. Miller, W. R. Heath, and J. Floege. 2001. Kidney protection against autoreactive $CD8^+$ T cells distinct from immunoprivilege and sequestration. *Kidney Int* 60: 664-671.
6. Bell, R. B., and L. Steinman. 1991. Trimolecular interactions in experimental autoimmune demyelinating disease and prospects for immunotherapy. *Semin Immunol* 3: 237-245.
7. Janeway jr., C. A., P. Travers, W. Mark, and S. Mark. 2002. Immunologie. *Berlin Spektrum Akademischer Verlag* 5. Auflage.
8. Heller, F., M. T. Lindenmeyer, C. D. Cohen, U. Brandt, D. Draganovici, M. Fischereder, M. Kretzler, H.-J. Anders, T. Sitter, I. Mosberger, D. Kerjaschki, H. Regele, D. Schlondorff, and S. Segerer. 2007. The contribution of B cells to renal interstitial inflammation. *Am J Pathol* 170: 457-468.
9. Kaissling, B., and M. Le Hir. 2008. The renal cortical interstitium: morphological and functional aspects. *Histochem Cell Biol* 130: 247-262.
10. Cheng, H. F., F. Nolasco, J. S. Cameron, G. Hildreth, G. H. Neild, and B. Hartley. 1989. HLA-DR display by renal tubular epithelium and phenotype of infiltrate in interstitial nephritis. *Nephrol Dial Transplant* 4: 205-215.
11. Wüthrich, R., L. Glimcher, M. Yui, A. Jevnikar, S. Dumas, and V. Kelley. 1990. MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. *Kidney Int* 37: 783-792.

12. Martin-Orozco, N., Y.-H. Wang, H. Yagita, and C. Dong. 2006. Cutting Edge: Programmed Death (PD) Ligand-1/PD-1 interaction is required for $CD8^+$ T cell tolerance to tissue antigens. *J Immunol* 177: 8291-8295.

13. Keir, M. E., G. J. Freeman, and A. H. Sharpe. 2007. PD-1 regulates self-reactive $CD8^+$ T cell responses to antigen in lymph nodes and tissues. *J Immunol* 179: 5064-5070.

14. Menke, J., J. A. Lucas, G. C. Zeller, M. E. Keir, X. R. Huang, N. Tsuboi, T. N. Mayadas, H. Y. Lan, A. H. Sharpe, and V. R. Kelley. 2007. Programmed death 1 ligand (PD-L) 1 and PD-L2 limit autoimmune kidney disease: distinct roles. *J Immunol* 179: 7466-7477.

CHAPTER 5

Renal tubular PD-L1 suppresses allo-reactive human T cell responses

Astrid Starke[1], Maja T. Lindenmeyer[1], Stephan Segerer[2], Matthias A. Neusser[3], Daniel M. Schmid[4], Clemens D. Cohen[1, 2], Rudolf P. Wüthrich[1, 2], Thomas Fehr[2] and Ying Waeckerle-Men[1]

[1]Institute of Physiology and Zürich Center for Integrative Human Physiology, University of Zürich, Switzerland;
[2]Division of Nephrology, [3] Division of Internal Medicine and [4]Department of Urology, University Hospital Zürich, Switzerland

Abstract

Renal proximal tubular epithelial cells (TECs) are targeted by infiltrating T cells during renal allograft rejection. The co-inhibitory molecules PD-L1 and PD-L2 are ligands of Programmed Death-1 (PD-1) and may protect TECs from T cell-mediated injury. In this study, we investigated the functional role of the PD-1/PD-L pathways in human renal allografts. In cultured human primary TECs the treatment with IFN-β and IFN-γ revealed a dose-dependent and synergistic increase of PD-L1 and PD-L2 expression. Their functional role was analyzed by human allogeneic T cell responses to TECs *in vitro*. Blockade of surface PD-L1, but not PD-L2, on TECs pre-treated with IFNs resulted in a significantly increased $CD4^+$ T cell proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells. PD-L1, PD-L2 and PD-1 mRNA and protein expression were upregulated in biopsies of patients with renal allograft rejection compared to pretransplant biopsies. PD-L1 induction was significantly associated with acute vascular rejection. In conclusion, our study suggests an inhibitory effect of the renal epithelial PD-1/PD-L1 pathway on alloreactive T cell responses. The upregulation of PD-L1 on TECs in patients with acute allograft rejection may reduce T cell-mediated injury.

Introduction

Acute renal allograft rejection remains a major obstacle for long-term allograft survival as it predisposes to chronic allograft injury with a progressive decline of renal function. Renal proximal tubular epithelial cells (TECs) are the main target of infiltrating alloreactive $CD4^+$ and $CD8^+$ T cells causing tubulointerstitial injury (1). The local inflammation induces MHC class I and class II expression on donor TECs, allowing recognition of allogeneic MHC/peptide complexes by recipient T cells (signal 1) (2). An additional signal 2 required for full T cell activation is provided by the binding of co-stimulatory molecules on APCs to their receptors on T cells (e.g. B7/CD28). In contrast a number of known co-inhibitory molecules downregulate T cell responses. PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are ligands for the Programmed-death 1 (PD-1, CD279) molecule which is induced on activated T and B cells. PD-L1 and PD-L2 are expressed on hematopoietic cells and parenchymal tissues such as heart, lung, liver, placenta and kidney (3, 4). Binding of PD-L1 or PD-L2 to PD-1 inhibits lymphocyte activation (3, 4).

The expression profile of co-stimulatory and co-inhibitory molecules on TECs is limited (5). While TECs do not express the typical co-stimulatory molecules B7.1 and B7.2 (6, 7), they express CD40 as a co-stimulatory molecule. PD-L1 is the most prominent co-inhibitory molecule on TECs. We and others previously observed that PD-L1 is strongly expressed in rejected murine kidney transplants, and that PD-L1 is markedly upregulated on cultured murine TECs after treatment with inflammatory cytokines (8, 9). Furthermore, high PD-L1 expression by murine TECs impairs antigen-specific $CD4^+$ and $CD8^+$ T cell responses (5, 8, 10). Several other studies demonstrated that the PD-1/PD-L1 pathway directly attenuates allograft rejection in experimental models of heart, skin and pancreas transplantation (11-13). Furthermore, PD-1 played a crucial role for the induction of $CD8^+$ T cell tolerance in an allogeneic mixed chimerism model (14).

The functional role of PD-1/PD-L pathways in human kidney allograft rejection is unknown. Thus, the goal of the present study was to investigate the function of PD-L1 and PD-L2 on human TECs in modulating alloreactive $CD4^+$ and $CD8^+$ T cell responses. Furthermore, expression of PD-L1, PD-L2 and PD-1 was evaluated in biopsies of kidney allografts with acute and chronic rejection.

Materials and Methods

General reagents

Cell culture reagents were obtained from Invitrogen (Gaithersburg, MD) and Sigma (St. Louis, MO). Human cytokines were purchased from R&D Systems (Oxford, UK).

Human cell lines and primary TEC cultures

The human renal TEC line HK-2 was cultured in K1 medium (15). Primary cultures of human TECs were generated from healthy parts of tumor nephrectomies from three patients (patient 1-3). Briefly, small fragments of cortical tissue were digested twice with type II collagenase (1 mg/ml in HBSS) (Invitrogen) for 90 min at 37°C. The cell suspension was passed through 40 μm sieves, washed and seeded on collagen-coated cell culture plates in K1 medium containing human EGF (5 ng/ml). Next day, after washing with HBSS; fresh K1 medium was added to allow primary TECs growing at 37°C in 5% CO_2 until confluence (5-7 days). Epithelial cell origin confirmed by positive cytokeratin staining was more than 95%. In functional assays, the 2nd passage of TECs with positive cytokeratin expression was used as APCs, which demonstrated a similar pattern of surface molecule expression and antigen-presentation capacity compared to the original primary TECs.

Flow cytometric analysis

Human TECs were harvested by trypsinization, washed and stained with mAb (all mouse anti-human) against PD-L1 (clone MIH1), PD-L2 (clone MIH18), HLA-ABC (clone W6/32) and HLA-DR (clone LN3). mAbs were purchased from eBioscience (San Diego, CA). Cells were analyzed using a FACSCantoII flow cytometer (BD Biosciences) and FlowJo software Version 7 (Tree Star, OR, USA).

Isolation and activation of human $CD4^+$ and $CD8^+$ T cells in vitro

Human PBMCs were isolated from heparinized whole blood of healthy volunteers (n=5) and of patient 2 (who provided the tissue for isolating TECs = P2 TEC) using Lymphoprep™ (AXIS-SHIELD PoC AS, Oslo, Norway) according to the manufacturer's protocol. PBMCs were washed with PBS three times followed by $CD4^+$ and $CD8^+$ T cell isolation with human CD8 and CD4 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. $CD4^+$ and $CD8^+$ T cells of healthy volunteers were then pre-activated either with PHA or allogeneic PBMCs to upregulate PD-1 expression on T cell surfaces:

1. PHA activation:

Isolated $CD4^+$ and $CD8^+$ T cells of healthy donors were adjusted to $2x10^6$cells/ml in IMDM medium supplemented with 10% FCS, seeded into 24-well-pates and incubated at 37°C, 5% CO_2. Activation of T cells was performed by adding 2 µg/ml of PHA for 5 days.

2. Allogeneic and autologous activation:

The T cell depleted fraction of PBMCs ($CD4^-CD8^-$PBMCs) of patient 2 was irradiated with 30 Gy and then co-cultured with purified $CD4^+$ or $CD8^+$ T cells of one healthy donor at a ratio of 2:1 (T cells:PBMCs) for 3 days in the presence of human IL-2 (5 ng/ml). As a control, $CD4^+$ and $CD8^+$ T cells of the same healthy donor were stimulated with their own $CD4^-CD8^-$ PBMCs (autologous stimulation) under the same conditions.

Alloreactive $CD4^+$ T cell proliferation induced by TECs

Human primary TECs were pre-treated with human IFN-β and IFN-γ (100 U/ml each) for 2 days. Cells were then harvested by trypsinization, washed and irradiated with 60 Gy. Irradiation did not influence the expression of PD-L1 and PD-L2 (data not shown). After washing, TECs were resuspended in IMDM and seeded in 96-well plates. TECs were pre-incubated with the relevant mAbs (PD-L1, PD-L2 or isotype control) for 1 h at 37°C prior to addition of pre-activated $CD4^+$ T cells at the indicated T cell/TEC ratios. T cell proliferation was determined after 72 h of co-incubation by using the non-radioactive colorimetric CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol. Results were presented as the value of the OD at 490 nm after subtraction of the OD values from medium alone and calculated as follows: $OD_{experimental} - OD_{TEC\ alone} - OD_{\ T\ cell\ alone}$.

Alloreactive $CD8^+$ T cell cytokine production stimulated by TECs

Human primary TECs were pre-treated with IFN-β and IFN-γ for 2 days, harvested and pre-incubated with the relevant mAbs or isotype control for 1 h, and then seeded in 96-well plates. Pre-activated human $CD8^+$ T cells were added at a responder/stimulator ratio of 30:1 ($3x10^5$ $CD8^+$ T cells to $1x10^4$ TECs) for 48 h.

Measurement of cytokines

Cytokine production was determined in the coculture supernatants after 48 h ($CD8^+$ T cell response) and 72 h ($CD4^+$ T cell response), respectively, either by human specific ELISA kits for IFN-γ or IL-2 (eBioscience, San Diego, USA) or by the BD™ CBA Flex Sets (BD Biosciences, San Jose, USA) according to the manufacturer's protocols.

Quantitative real-time PCR of renal biopsies

Human renal biopsy specimens were procured in an international multicenter study, the European Renal cDNA Bank-Kroener-Fresenius Biopsy Bank (ERCB-KFB). Biopsies were obtained from patients after informed consent and with approval of the local ethics committees. Clinical characteristics of all patients are shown in Table I. Tubulointerstitial tissue was prepared and analyzed by real-time RT-PCR as reported earlier (16). Pre-developed TaqMan reagents were used for human PD-L1 (CD274) [NM_014143.2], PD-L2 (CD273, PDCD1LG2) [NM_025239.2], and PD-1 (CD279, PDCD1,) [NM_005018.1] as well as the housekeeper genes (GAPDH and 18SrRNA; Applied Biosystems). The mRNA expression was analyzed by standard curve quantification. Normalization to both housekeeper genes gave comparable results; data based on 18S rRNA are shown.

Immunohistochemistry

Immunohistochemistry was performed as previously described (17). The biopsies were archival materials collected retrospectively at the University of Vienna (2006-2008). In brief, sections were dewaxed in xylene, rehydrated in a series of graded ethanols, incubated in 3% hydrogen peroxide (to block endogenous peroxidases). To block endogenous biotin the Avidin/Biotin blocking Kit (Vector, Burlingame, CA) was used. Ag retrieval was performed in an autoclave oven in Ag retrieval solution. Incubation with the primary Ab was performed for 1 h or overnight. Incubation with biotinylated secondary Ab was for 30 min, followed by the ABC reagent. For PD-1, two consecutive secondary Abs were used to enhance the signal. 3'3'Diaminobenzidine (DAB, Sigma, Taufkirchen, Germany) with metal enhancement (resulting in a black color product) served as detection system. The rabbit polyclonal Ab against PD-L1 (ab41890, Abcam, Cambridge, UK) was used in a dilution of 0.5 µg/ml (1:400 in 10 % non-fat dry milk). The Ab was raised against a peptide corresponding to amino-acids 144-291 of human PD-L1. The peptide was used to block the signal via pre-incubation of the Ab as additional control. Furthermore non-immune rabbit serum was used as a negative control. The mAb mouse anti-human PD-1 (clone MIH4, Natutec, Frankfurt am Main, Germany) was used in a dilution of 40 µg/ml. Isotype matched mouse IgG1 was used as negative control.

Statistics

All *in vitro* cell experiments were performed in triplicates and were repeated at least twice. Data are expressed as mean ± SD. The unpaired Student's t test was used to analyze all data

of the *in vitro* experiments. Mann Whitney test was used to perform statistical analysis of PD-L1, PD-L2 and PD-1 mRNA expression in human biopsies. Additional Bonferroni correction was applied to assess for multiple testing for the mRNA levels. Pearson's correlation was done to correlate mRNA expressions to the clinical characteristics. Significance was accepted at $p \leq 0.05$.

Results

PD-L1 and PD-L2 surface expression on human renal TECs upon IFN-β and IFN-γ treatment in vitro

To explore the functional role of PD-L1 and PD-L2 on human TECs, we first studied the expression pattern on cultured TECs. TECs were stimulated with human IFN-β and IFN-γ for 48 h. PD-L1 and PD-L2 were constitutively expressed on TECs (Fig. 1A). Treatment with either IFN-β or IFN-γ was able to further upregulate the expression of both PD-L1 and PD-L2 in a dose dependent manner (Fig. 1A). Furthermore, stimulation of TECs with both INFs revealed a synergistic effect (Fig. 1B). We performed these experiments with primary TECs (n=3) and HK-2 cells and obtained similar results (data not shown).

Stimulation with IFN-β and IFN-γ also strongly upregulated constitutive MHC class I expression (Fig 1C). MHC class II molecules were not constitutively expressed on TECs (Fig. 1C), but stimulation with IFN-γ led to a marked increase. IFN-β had no effect.

PD-L1 on TECs inhibited proliferation and cytokine production of $CD4^+$ T cells

In these experiments, alloreactive $CD4^+$ T cell proliferation was performed by incubating PHA-activated human $CD4^+$ T cells with IFN-pretreated human TECs. Anti-PD-L1 and -PD-L2 mAbs or isotype controls were included to examine the function of renal epithelial PD-L1 and PD-L2. As shown in Fig. 2A, compared to the anti-CD3 mAb-induced $CD4^+$ T cell proliferation, TECs generated from nephrectomized kidneys of 2 patients (P1 and P2 TEC) induced a very weak T cell proliferation. Importantly, double blockade of PD-L1 and PD-L2 on both P1 and P2 TECs significantly increased the $CD4^+$ T cell proliferation and cytokine production (Fig. 2A and 2C). To further define whether the enhancement of T cell proliferation was mediated via the blockade of PD-L1, PD-L2 or both, single blockade was performed. Interestingly, inhibition of PD-L1 alone was able to augment $CD4^+$ T cell proliferation, which was comparable to the responses obtained with double blockade. In contrast, PD-L2 blockade was not effective (Fig. 2B).

The cytokine profile in the supernatants of $CD4^+$ T cell/TECs co-cultures was investigated, including IL-2, IL-4, IL-10, IFN-γ and TNF-α. Similar to the T cell proliferation, TECs stimulated negligible IFN-γ production comparable to the baseline level of IFN-γ production by $CD4^+$ T cells alone (Fig. 2C). Inhibition of PD-L1 on TECs significantly increased the IFN-γ production up to 5-10-fold. Again, the same magnitude of IFN-γ secretion was seen when both PD-L1 and PD-L2 on TECs were blocked, whereas inhibition of PD-L2 alone had no effect. TNF-α secretion by $CD4^+$ T cells was also observed only after PD-L1, but not PD-L2 blockade (data not shown). Although proliferation of $CD4^+$ T cells after blockade of PD-L1 on TECs could be measured, IL-2 production was not detectable in the supernatants of $CD4^+$ T cell/TECs co-culture after 72 h (data not shown). In addition, $CD4^+$ T cells did not produce IL-4 or IL-10 after stimulation with TECs under these experimental conditions (data not shown).

PD-L1 on TECs inhibited cytokine production of $CD8^+$ T cells

To study the alloreactive $CD8^+$ T cell response after stimulation with TECs, TECs pre-treated with IFNs were used as stimulators to test the IFN-γ production by PHA-activated $CD8^+$ T cells. Similarly to the $CD4^+$ T cell responses, TECs alone only induced weak $CD8^+$ T cell activation, measured by a low IFN-γ production in the supernatants of T cell/TECs co-cultures. Blockade of both PD-L1 and PD-L2 on the TECs significantly increased the IFN-γ production of $CD8^+$ T cells by 2-3 folds (Fig. 3). In analogy to the $CD4^+$ T cell responses, single PD-L1 blocking on TECs increased the IFN-γ production of $CD8^+$ T cells to the same magnitude as with the double blocking, and no increase of IFN-γ production was obtained by PD-L2 blocking (Fig. 3).

PD-L1 on TECs inhibited the IFN-γ production of alloreactive $CD4^+$ and $CD8^+$ T cells

Since PHA activates T cells non-specifically and independently of TCR signaling, our results may not be completely transferable to alloreactive T cell responses. Thus we repeated these experiments by using allogeneic and autologous pre-stimulation of $CD4^+$ and $CD8^+$ T cells. Autologously and allogeneically pre-stimulated $CD4^+$ and $CD8^+$ T cells were co-cultured with IFN pre-treated TECs of patient 2 (P2 TEC). Since blockade of PD-L2 on TECs did not increase the T cell response in the previous experiments (Fig. 2 and 3), only PD-L1 blockade was performed. Allogeneic pre-activation of $CD4^+$ T cells with T cell-depleted PBMCs of patient 2 induced high amounts of IFN-γ compared to autologous pre-activation of $CD4^+$ T cells (Fig. 4A, T cells alone), indicating that the allogeneic pre-stimulation effectively

activated $CD4^+$ T cells of the healthy donor.

Re-stimulation of these allogeneically or autologously pre-activated $CD4^+$ T cells with the TECs of this patient 2 (P2 TEC) could not further increase the IFN-γ secretion. However, inhibition of PD-L1 on P2 TEC significantly augmented the IFN-γ production by allogeneically and to a much lesser degree also by autologously pre-activated $CD4^+$ T cells. As expected, the cytokine response of the allogeneically pre-activated $CD4^+$ T cells was significantly higher.

The alloreactive $CD8^+$ T cell response showed a similar pattern (Fig. 4B). Pre-activation of $CD8^+$ T cells by allogeneic $CD4^-CD8^-$PBMC of patient 2 induced moderate IFN-γ production. Inhibition of PD-L1 on P2 TEC dramatically increased the IFN-γ secretion of allogeneically pre-activated $CD8^+$ T cells. In contrast, blockade of PD-L1 on P2 TEC only slightly enhanced the IFN-γ production of autologously pre-activated $CD8^+$ T cells. In conclusion, these data reveal that $CD4^+$ and $CD8^+$ T cells pre-activated with the patient's allogeneic $CD4^-CD8^-$ PBMCs showed a much stronger cytokine response to P2 TECs than autologously pre-activated T cells. Our results suggest that PD-L1 on TECs plays a predominant role in inhibiting alloreactive $CD4^+$ and $CD8^+$ T cell responses induced by TECs.

Strong induction of PD-L1, PD-L2 and PD-1 mRNAs in human biopsies of kidney allograft rejection

The mRNA expression of PD-L1, PD-L2 and their receptor PD-1 was then investigated in renal biopsies of patients with acute kidney transplant rejection (ATR), interstitial fibrosis/tubular atrophy (IF/TA) (18) and BK virus nephropathy (BKN) by real-time RT-PCR. Compared to control tissues (pretransplant biopsies; living donor n=9, deceased donor n=1), PD-L1 mRNA transcript levels were significantly increased only in renal biopsies of patients with ATR, but not in biopsy samples with IF/TA or BKN (Fig. 5A). PD-L2 mRNA was significantly enhanced in biopsies of patients with ATR or IF/TA (Fig. 5B). In addition, biopsies of all patients showing a high PD-L1 mRNA induction had also increased mRNA levels of PD-L2 (Pearson, r=0.6480, p=0.0006). PD-1 mRNA was also significantly induced in biopsies with ATR, IF/TA and BKN (Fig. 5C).

Patient characteristics and correlation to the PD-L1, PD-L2 and PD-1 mRNA levels

The relevant clinical parameters including recipient age, gender, creatinine and proteinuria as well as histological parameters like scores for tubulitis, interstitial infiltration, chronic lesions

and Banff classification at the time of biopsy were extracted from the ERCB-KFB (Table I). PD-L1, PD-L2 and PD-1 mRNA revealed no correlation to creatinine and proteinuria levels. Possibly the cohort size was too small for such analysis. Since, follow-up data are not routinely collected from allograft recipients in the ERCB-KFB, a correlation with the response to treatment could not be assessed. However, the expression of PD-L1 was significantly increased in patients with vascular rejection (Banff IIA and IIB) compared to tubulointerstitial rejection (Banff IA and IB) (Fig. 6A). This was not the case for PD-L2 (data not shown). In contrast, the score for tubulitis showed a tendency to be lower in patients with vascular rejection (Banff IIA and IIB, Fig. 6B).

PD-L1 and PD-1 localization in human allograft rejection

We speculated that the increased mRNA expression of PD-1 detected in the biopsies might be due to infiltrating lymphocytes and the enhanced PD-L1 mRNA expression would be expected on TECs as well as on infiltrating leukocytes. To localize PD-L1 and PD-1 we performed immunohistochemistry on a series of archival allograft biopsies and on two renal allograft nephrectomies with severe acute vascular rejection in addition to signs of chronic injury (IF/TA, n=2). The allograft biopsies included acute interstitial rejection (Banff IB) and biopsies without acute rejection with well preserved tissue architecture (n=3). In allograft nephrectomies, surface expression of PD-L1 was found on infiltrating cells in the tubulointerstitium which displayed dendritic cell morphology (Fig. 7A) and on tubular epithelial cells (Fig. 7A and 7C). Pre-incubation of the anti PD-L1 antibody with its peptide completely abolished the positive PD-L1 staining (Fig. 7B). PD-1 was expressed by interstitial infiltrating lymphocytes but not on tubular cells (Fig. 7D).

Discussion

The wide distribution of PD-L1 and PD-L2 in lymphoid and parenchymal tissues (3, 4) suggests a broad regulatory function on various immune responses, including autoimmunity and self tolerance (13). Recent studies indicated an important role for the PD-1/PD-L pathway for allograft rejection and tolerance in experimental models (11, 19). Here, we demonstrated that the co-inhibitory molecule PD-L1 expressed on human renal TECs suppresses human alloreactive T cell responses and is associated with acute kidney allograft rejection.

Earlier studies showed that $CD4^+$ T cells do not respond to allogeneic TECs pre-treated with IFN-γ. This was in part due to the lack of co-stimulation, especially the absence of B7.1 molecules on TECs (6, 20). However, our study now reveals that the existence of the PD-1/PD-L1 co-inhibitory pathway plays a crucial role for the unresponsiveness of alloreactive T cells towards renal TECs, since inhibition of PD-L1 on TECs strongly augmented the $CD4^+$ and $CD8^+$ T cell responses. An increased alloreactive $CD8^+$ T cell response could only be measured in terms of IFN-γ production, whereas the same $CD8^+$ T cells were not able to lyse allogeneic renal TECs even though they demonstrated a strong CTL activity to Jurkat cells (data not shown). These results indicate that human TECs are highly resistant to the attack of alloreactive CTLs *in vitro*. The limited co-stimulatory molecule expression on TECs, including the lack of strong B7/CD28 co-stimulation, could at least partially explain this phenomenon.

Our results demonstrated that IFN-β and IFN-γ play a critical role in the upregulation of the PD-L1 and PD-L2 expression on TECs *in vitro*. Obata *et al.* showed that the expression of IFN-γ mRNA was strongly increased in acute and chronic human renal allograft biopsies (21). It is therefore possible that, during local inflammation, IFN-β production as part of the innate immune response and IFN-γ secretion from infiltrating alloreactive T cells are both responsible for the *in vivo* increase of PD-L1 (and PD-L2) expression on renal tubulointerstitial tissues as observed in our study. This is consistent with the observation that the expression of PD-1 is absent in healthy kidneys without inflammation (22). The expression of PD-1 in allograft biopsies with acute rejection indicates the presence of activated T cells within the inflamed kidneys. Importantly, more intense tubulitis seems to be associated with lower PD-L1 expression during tubulointerstitial rejection, whereas the opposite was observed for vascular rejections. This may suggest that during more severe acute vascular rejections the tubulointerstitium is partially protected by the expression of PD-L1,

whereas this is not the case for the vascular endothelium which does not express PD-L1 (Fig. 7A). These results complement previous observations of high PD-L1 expression on renal tubules in other immune-mediated human renal diseases, including diffuse proliferative lupus nephritis, IgA nephropathy and tubulointerstitial nephritis (9, 22). Therefore there is increasing evidence that the PD-1/PD-L1 pathway is involved in immune-mediated human renal diseases and especially in acute kidney transplant rejection.

Although PD-L2 upregulation was observed in our biopsy samples and on IFN-stimulated TECs, it does not seem to play an inhibitory role in human kidney transplantation. There was no correlation to histological parameters and *in vitro* blockade of PD-L2 on TECs was not effective. This was not due to the failure of the anti-human PD-L2 mAb included in this study because the same clone of mAb was successfully used to inhibit PD-L2 activity of T cell stimulator cells by others (23). Several studies suggested distinct roles of PD-L1 and PD-L2 in regulating T cell activity. Tanaka *et al*. indicated that blockade of PD-L1 but not PD-L2 accelerated cardiac allograft rejection associated with an increased frequency of IFN-γ producing alloreactive T cells and the expansion of effector $CD8^+$ T cells (24). Consistent with this observation, Sandner *et al.* showed that inhibition of PD-L1 but not PD-L2 enhanced $CD4^+$ T cell activation in a skin graft model (12). The function of PD-L2 may also depend on the location of immune responses. Habicht *et al.* demonstrated that in the host lymphoid organs as opposed to parenchymal tissue the *in vivo* activation of both alloreactive $CD4^+$ and $CD8^+$ T cells was primarily mediated by PD-L2. Furthermore, the increased proliferation of $CD8^+$ T cells after *in vivo* blockade of PD-L2 was dependent on CD28-mediated co-stimulation signal (25), which does not play a role for TECs, that do not express B7 molecules.

Experimental models of heart (11, 19) and corneal allografts (26) showed a critical and protective role of the PD-1/PD-L1 pathway in transplantation. Although the increase of PD-L1 on donor TECs is not sufficient to completely prevent acute allograft rejection, our results suggest a protective mechanism of parenchymal tissues to mitigate alloreactive human T cell responses and tissue injury via PD-1/PD-L1 co-inhibitory pathway. Strategies to selectively enhance PD-L1 expression on TECs as well as on professional APC might be therapeutically useful to prevent acute kidney transplant rejection.

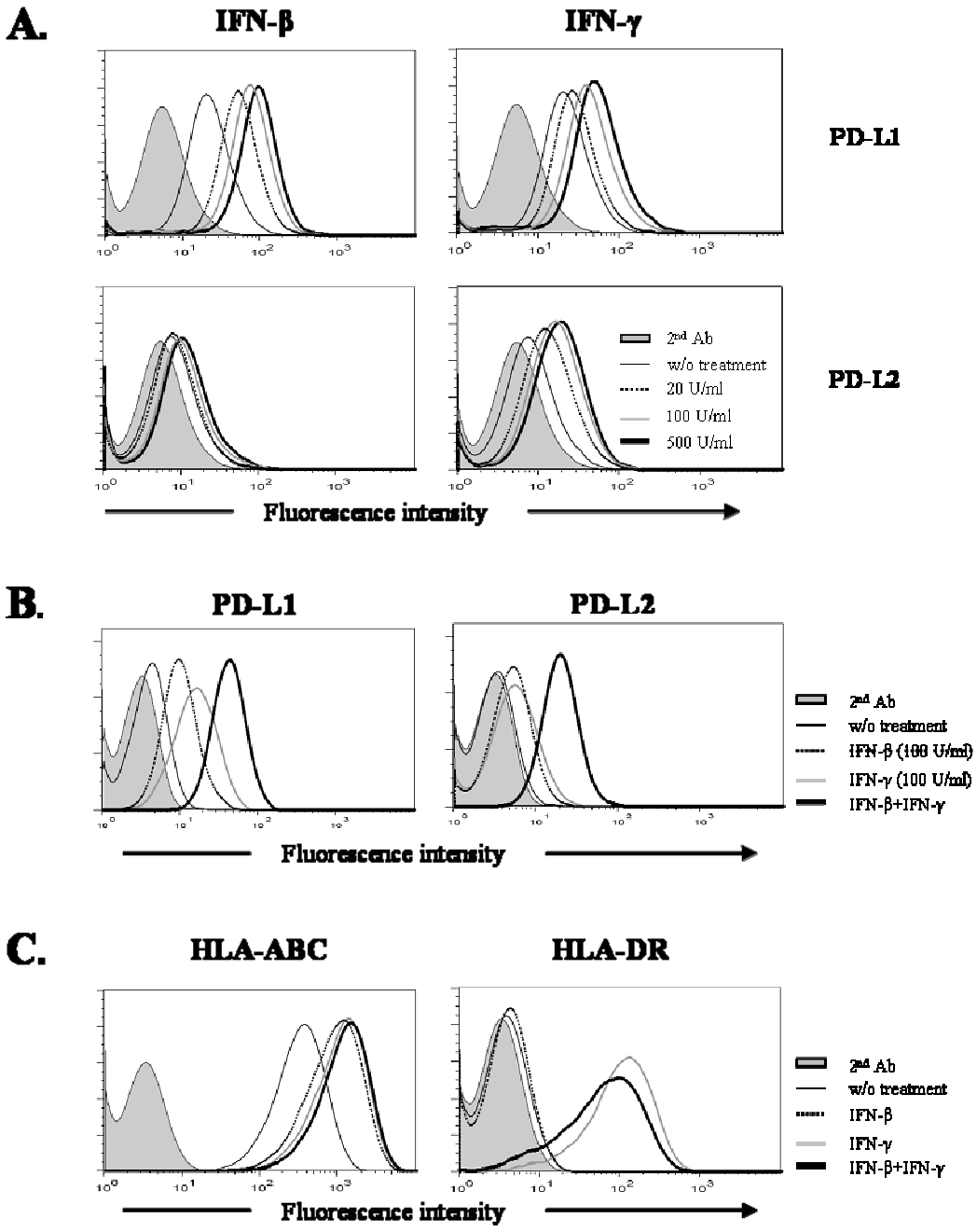

Figure 1: Flow cytometric analysis of PD-L1/2 and MHC class I/II on human primary TECs. (A, B) Human primary TECs were stained with mAb for PD-L1 and PD-L2 expression after 48 h stimulation with IFN-β or IFN-γ at different doses (A) as well as after treatment with both cytokines together (B) and compared to untreated controls. (C) MHC class I and MHC class II expression was determined with Ab against HLA-ABC and HLA-DR, respectively. The results are representative for the three patients' TECs as well as the cell line HK-2.

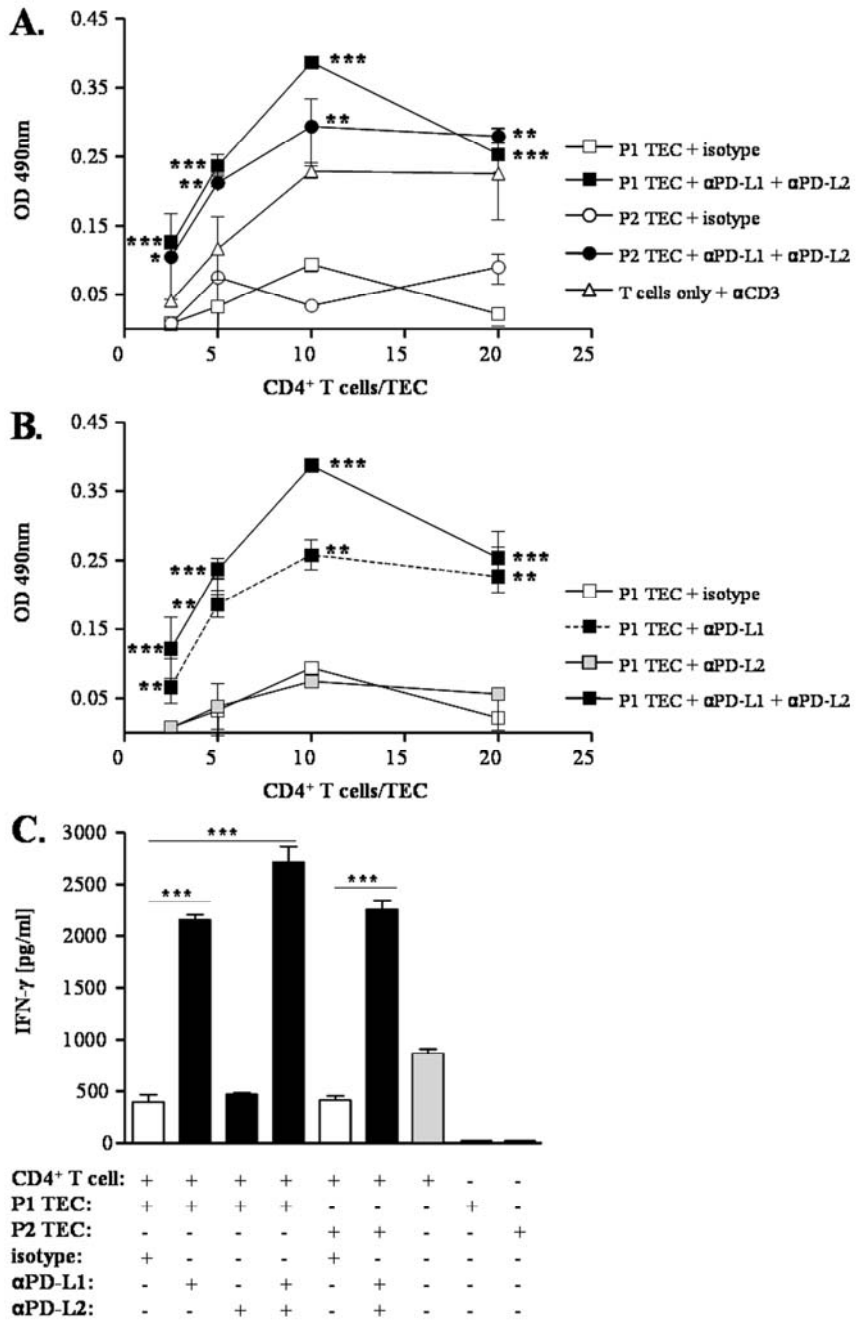

Figure 2: Proliferation and IFN-γ production of CD4$^+$ T cells after stimulation with human primary TECs pre-treated with IFNs. CD4$^+$ T cells activated with PHA were co-cultured with IFN-pre-treated TECs of patient 1 (P1 TEC) or of patient 2 (P2 TEC) (2x10^4 cells/well) at the indicated responder/stimulator ratios of 20, 10, 5 and 2.5:1. Before addition of TECs to T cells, TECs were incubated for 1 h with αPD-L1 and/or αPD-L2 Ab or isotype control. After 72 h of co-culture the proliferation (A, B) and the IFN-γ production (C) of CD4$^+$ T cells was determined. The results represent mean values ± SD of triplicate wells and are representative for CD4$^+$ T cell responses of 4 different donors which gave similar results. *p<0.05, **p<0.001, ***p<0.0001 when compared with isotype control (unpaired Student's t test).

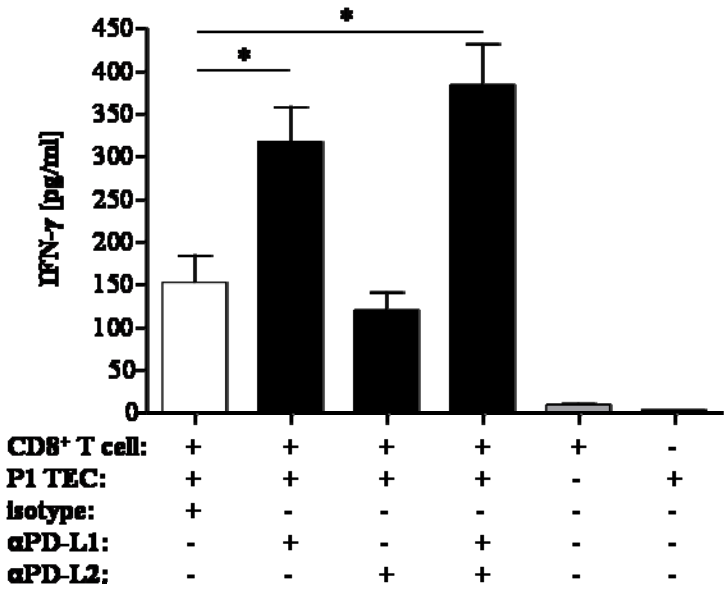

Figure 3: IFN-γ production of CD8$^+$ T cells after stimulation with human primary TECs pre-treated with IFNs. CD8$^+$ T cells activated with PHA were co-cultured with IFN-pre-treated TECs of patient 1 (P1 TEC) (1x10^4 cells/well) at the responder/stimulator ratio of 30:1. Before addition of TECs to CD8$^+$ T cells, TECs were incubated with αPD-L1 and/or αPD-L2 Ab or isotype control. After 48 h of co-culture the IFN-γ production of CD8$^+$ T cells was determined. The results represent mean values ± SD of triplicate wells and are representative for CD8$^+$ T cell responses of 4 different responders which gave similar results. *$p<0.05$, when compared with isotype control (unpaired Student's t test).

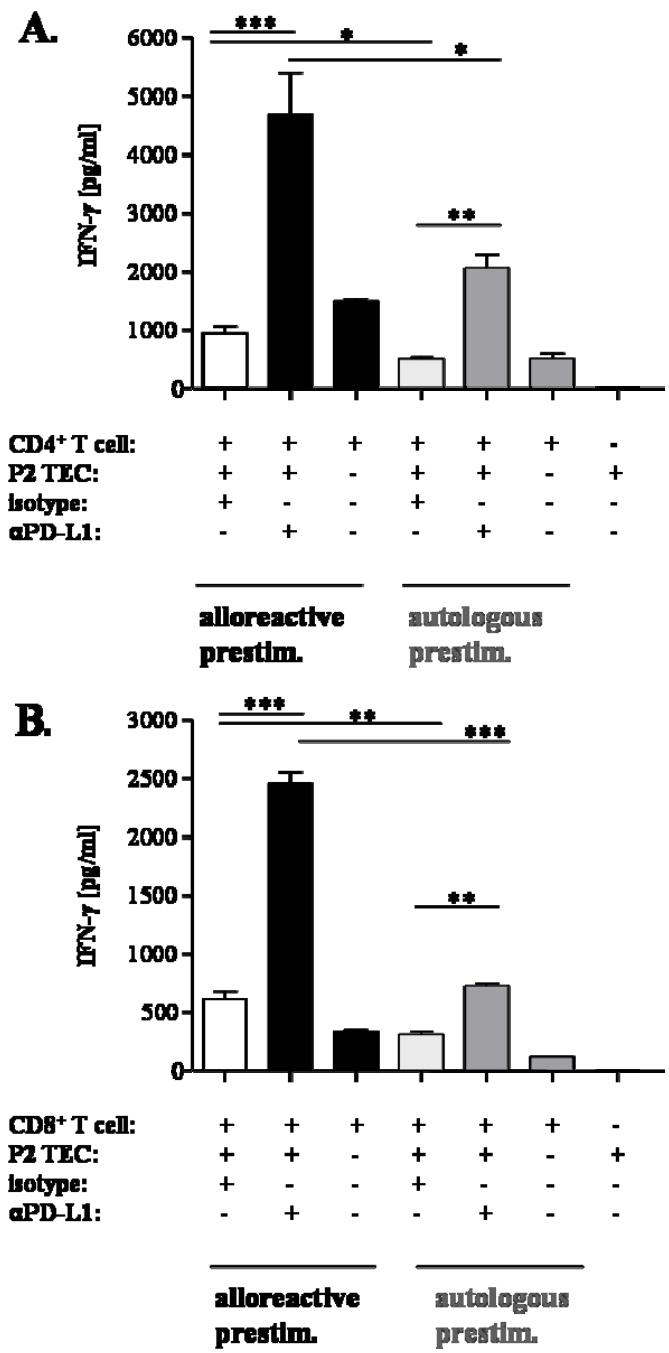

Figure 4: IFN-γ production of alloreactive CD4$^+$ and CD8$^+$ T cells after stimulation with human primary TECs pre-treated with IFNs. CD4$^+$ and CD8$^+$ T cells were pre-activated with allogeneic (patient 2) or autologous T cell-depleted PBMCs and were co-cultured with IFN-pre-treated TECs of patient 2 (P2 TEC) at a responder/stimulator ratio of 20 and 30 for the CD4$^+$ T cells and the CD8$^+$ T cells, respectively. Before addition of TECs to T cells, TECs were incubated for 1 h with αPD-L1 Ab or isotype control. After 72 h (A) or 48 h (B) of co-culture the IFN-γ production of CD4$^+$ T cells (A) and CD8$^+$ T cells (B) was determined. *p<0.05, **p<0.001, ***p<0.0001 when compared with isotype control (unpaired Student's t test).

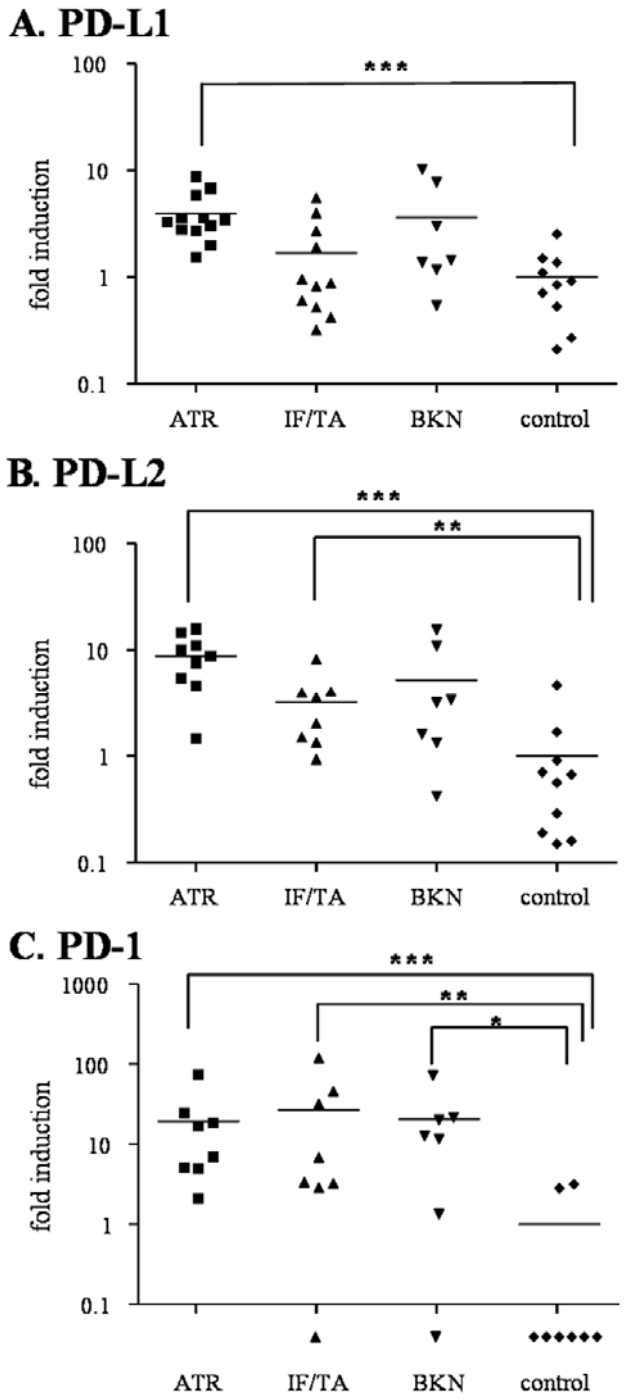

Figure 5: PD-L1, PD-L2 and PD-1 mRNA expression in human renal biopsies. (A) Renal biopsies from patients with acute transplant rejection (ATR, n=12), interstitial fibrosis and tubular atrophy (IF/TA n=11) and BK virus nephropathy (BKN, n=7) were compared to pre-transplant tissue samples (control, n=10) for the mRNA expression of PD-L1. (B) For PD-L2 9 samples of ATR, 8 of IF/TA, 7 of BKN and 10 of controls and (C) for PD-1 8 of ATR, 8 of IF/TA, 7 of BKN and 8 of controls were analyzed. mRNA expression of PD-L1, PD-L2 and PD-1 were normalized to the expression of 18S rRNA and the fold inductions of the target genes in the above mentioned entities were calculated compared to the control tissue (=1).

PD-1 mRNA expression was not detectable in some samples which are shown below the x-axis. *p<0.05, **p<0.001, ***p<0.0001 when compared with controls (Mann-Whitney-U Test and Bonferroni correction).

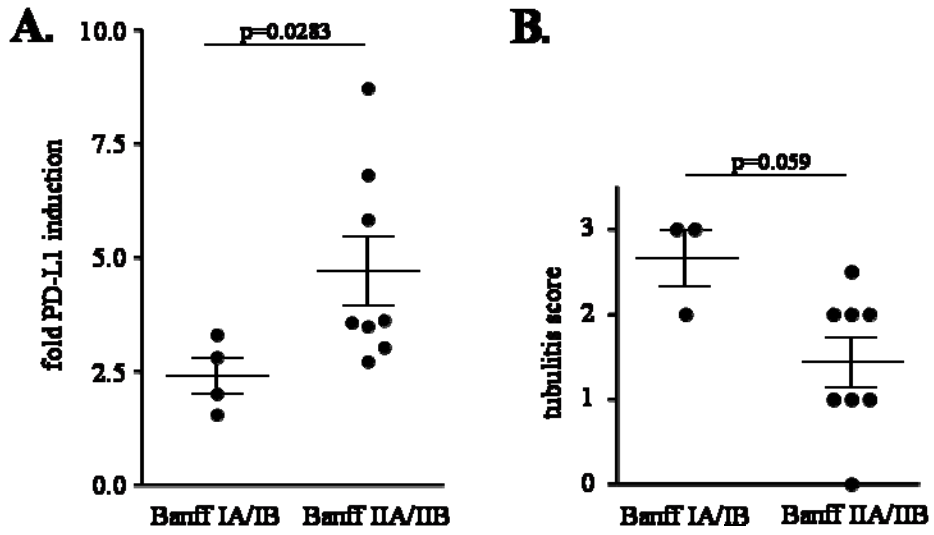

Figure 6: PD-L1 mRNA induction is pronounced in vascular allograft rejection. (A) Relation of PD-L1 induction between patients suffering from acute tubulointerstitial rejection (Banff IA/IB) and vascular rejection (Banff IIA/IIB). (B) Comparison of the tubulitis score between tubulointerstitial rejection (Banff IA/IB) and vascular rejection (Banff IIA/IIB) (Mann-Whitney-U Test).

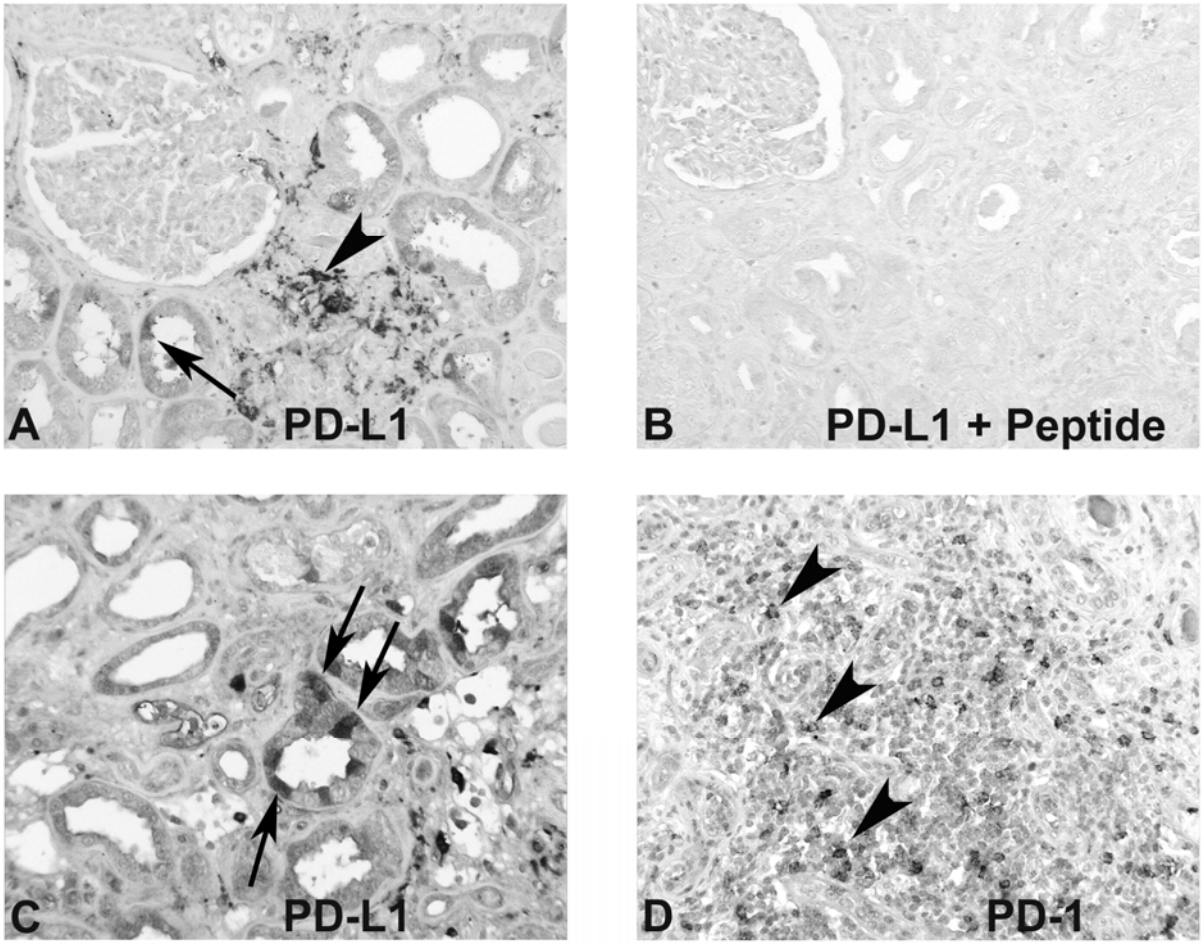

Figure 7: Immunohistochemistry of PD-L1 and PD-1 expression on allograft biopsies. (A) In allograft nephrectomies PD-L1 was expressed by large cells with a dendritic cell morphology in the tubulointerstitium (arrowheads) as well as by some tubular epithelial cells (arrows). (B) Pre-incubation of the anti- PD-L1 Ab with the peptide used for immunization completely abolished the positive staining demonstrating specificity of the staining. (C) Prominent expression of PD-L1 by tubular epithelial cells (arrows). (D) PD-1 positive cells resemble small lymphocytes (some are indicated by arrowheads), which were present in foci of smaller and larger accumulations of infiltrating cells. (original magnifications: x100 A, B; x200 C, D)

Table I: Clinical and histological characteristics of biopsies from patients with acute transplant rejection, interstitial fibrosis/tubular atrophy, BK virus nephropathy and control subjects analyzed by real-time RT-PCR.

Biopsy group	Age (yr)	Gender	Graft	Creatinine (µmol/l)	Proteinuria (g/d)	Grade of damage			Banff
						Ci/ct	int if	tub inf	
Acute transplant rejection									
ATR1	47	M	dcd	424	n.a.	2	3	2-3	IIB
ATR2	71	F	dcd	455	0.5	2	1-2	0	IIA
ATR3	47	M	dcd	240	0.1	0	2	2	IIA
ATR4	49	M	dcd	720	n.a.	0	2	1	IIA
ATR5	61	F	dcd	456	n.a.	0	2	3	IB
ATR6	54	F	dcd	909	n.a.	0	2	2	IA
ATR7	49	M	dcd	177	0.18	2	n.a.	n.a.	IA
ATR8	47	M	dcd	440	0.3	0-1	2	3	IB
ATR9	65	M	dcd	488	n.a.	2	2	2	IIA
ATR10	61	F	dcd	637	n.a.	1	2	1	IIB
ATR11	43	M	dcd	908	n.a.	0	2	1	IIB
ATR12	49	M	n.a.	203	n.a.	0	1	2	IIA
Interstitial fibrosis/ Tubular atrophy									
IF/TA1	46	M	dcd	174	n.a.	1	0-1	0	
IF/TA2	60	F	n.a.	220	0.325	1	0-1	0	
IF/TA3	23	F	liv	188	n.a.	1	0-1	0	
IF/TA4	66	F	dcd	120	2	1-2	0	0	
IF/TA5	32	F	dcd	234	n.a.	3	1	0	
IF/TA6	52	n.a.	n.a.	548	1.82	2	0-1	0	
IF/TA7	37	M	n.a.	884	7	2	0	0	
IF/TA8	32	M	dcd	171	1.5	1-2	0-1	0	
IF/TA9	34	F	dcd	495	0.8	3	0-1	0	
IF/TA10	67	M	dcd	294	0.2	1	0-1	0	
IF/TA11	66	M	dcd	225	5	1	0-1	0	
BK virus nephro-pathy									
BKN1	37	M	dcd	301	n.a.	0-1	2	0	
BKN2	41	M	dcd	539	0.4	2	1-2	3	
BKN3	52	F	dcd	273	8.3	0	2	2	
BKN4	35	F	dcd	528	1.26	2	1	0	
BKN5	57	M	dcd	426	n.a.	1	2	2	
BKN6	66	F	dcd	320	0	3	1-2	0	
BKN7	40	F	dcd	280	2.3	2	1-2	0-1	
Control subjects									
C1	35	F	liv	<97	<0.2				
C2	39	M	liv	<97	<0.2				
C3	55	F	liv	<97	<0.2				
C4	41	M	liv	<97	<0.2				
C5	61	M	liv	<97	<0.2				
C6	58	F	liv	<97	<0.2				
C7	27	M	liv	<97	<0.2				
C8	n.a.	n.a.	dcd	<97	<0.2				
C9	54	F	liv	<97	<0.2				
C10	61	F	liv	<97	<0.2				

ci/ct: chronic interstitial fibrosis and chronic tubular atrophy; int inf: interstitial infiltration/inflammation; tub inf: tubular infiltration/tubulitis; Grade of damage/score **0:** no or minimal, <5% for ci/ct and < 10% for int inf; **1:** mild (<25%); **2**: moderate <50%; **3**: severe > 50%; for tub inf **0:** no cells/tubular cross section; **1:** 1 to 4 cells/tubular cross section; **2:** 5 to 10 cells/tubular cross section; **3:** >10 cells/tubular cross section; Banff IA/B: tubulointerstitial rejection; IIA/B-vascular rejection; n.a: not available; dcd: deceased donor; liv: living donor

References

1. Cornell, L. D., R. N. Smith, and R. B. Colvin. 2008. Kidney transplantation: mechanisms of rejection and acceptance. *Annu Rev Pathol* 3: 189-220.
2. Andersen, C. B., S. D. Ladefoged, and S. Larsen. 1994. Acute kidney graft rejection. A morphological and immunohistological study on "zero-hour" and follow-up biopsies with special emphasis on cellular infiltrates and adhesion molecules. *Apmis* 102: 23-37.
3. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser, L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 Family member leads to negative regulation of lymphocyte activation. *J Exp Med* 192: 1027-1034.
4. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe, and G. J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2: 261-268.
5. Waeckerle-Men, Y., A. Starke, P. R. Wahl, and R. P. Wüthrich. 2007. Limited costimulatory molecule expression on renal tubular epithelial cells impairs T cell activation. *Kidney Blood Press Res* 30: 421-429.
6. Frasca, L., F. Marelli-Berg, N. Imami, I. Potolicchio, P. Carmichael, G. Lombardi, and R. Lechler. 1998. Interferon-gamma-treated renal tubular epithelial cells induce allospecific tolerance. *Kidney Int* 53: 679-689.
7. de Haij, S., A. M. Woltman, L. A. Trouw, A. C. Bakker, S. W. Kamerling, S. W. van der Kooij, L. Chen, R. A. Kroczek, M. R. Daha, and C. van Kooten. 2005. Renal tubular epithelial cells modulate T-cell responses via ICOS-L and B7-H1. *Kidney Int* 68: 2091-2102.
8. Schoop, R., P. Wahl, M. Le Hir, U. Heemann, M. Wang, and R. P. Wüthrich. 2004. Suppressed T-cell activation by IFN-γ-induced expression of PD-L1 on renal tubular epithelial cells. *Nephrol Dial Transplant* 19: 2713-2720.
9. Chen, Y., J. Zhang, J. Li, L. Zou, T. Zhao, Y. Tang, and Y. Wu. 2006. Expression of B7-H1 in inflammatory renal tubular epithelial cells. *Nephron Exp Nephrol* 102: e81-92.
10. Waeckerle-Men, Y., A. Starke, and R. P. Wüthrich. 2007. PD-L1 partially protects

renal tubular epithelial cells from the attack of $CD8^+$cytotoxic T cells. *Nephrol Dial Transplant* 22: 1527-1536.

11. Koga, N., J.-i. Suzuki, H. Kosuge, G. Haraguchi, Y. Onai, H. Futamatsu, Y. Maejima, R. Gotoh, H. Saiki, F. Tsushima, M. Azuma, and M. Isobe. 2004. Blockade of the interaction between PD-1 and PD-L1 accelerates graft arterial disease in cardiac allografts. *Arterioscler Thromb Vasc Biol* 24: 2057-2062.
12. Sandner, S. E., M. R. Clarkson, A. D. Salama, A. Sanchez-Fueyo, C. Domenig, A. Habicht, N. Najafian, H. Yagita, M. Azuma, L. A. Turka, and M. H. Sayegh. 2005. Role of the Programmed Death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol* 174: 3408-3415.
13. Keir, M. E., S. C. Liang, I. Guleria, Y. E. Latchman, A. Qipo, L. A. Albacker, M. Koulmanda, G. J. Freeman, M. H. Sayegh, and A. H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203: 883-895.
14. Haspot, F., T. Fehr, C. Gibbons, G. Zhao, T. Hogan, T. Honjo, G. J. Freeman, and M. Sykes. 2008. Peripheral deletional tolerance of alloreactive CD8 but not CD4 T cells is dependent on the PD-1/PD-L1 pathway. *Blood* 112: 2149-2155.
15. Wüthrich, R., L. Glimcher, M. Yui, A. Jevnikar, S. Dumas, and V. Kelley. 1990. MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. *Kidney Int* 37: 783-792.
16. Cohen, C. D., K. Frach, D. Schlondorff, and M. Kretzler. 2002. Quantitative gene expression analysis in renal biopsies: a novel protocol for a high-throughput multicenter application. *Kidney Int* 61: 133-140.
17. Segerer, S., F. Heller, M. T. Lindenmeyer, H. Schmid, C. D. Cohen, D. Draganovici, J. Mandelbaum, P. J. Nelson, H. J. Grone, E. F. Grone, A. M. Figel, E. Nossner, and D. Schlondorff. 2008. Compartment specific expression of dendritic cell markers in human glomerulonephritis. *Kidney Int* 74: 37-46.
18. Solez, K., R. B. Colvin, L. C. Racusen, B. Sis, P. F. Halloran, P. E. Birk, P. M. Campbell, M. Cascalho, A. B. Collins, A. J. Demetris, C. B. Drachenberg, I. W. Gibson, P. C. Grimm, M. Haas, E. Lerut, H. Liapis, R. B. Mannon, P. B. Marcus, M. Mengel, M. J. Mihatsch, B. J. Nankivell, V. Nickeleit, J. C. Papadimitriou, J. L. Platt, P. Randhawa, I. Roberts, L. Salinas-Madriga, D. R. Salomon, D. Seron, M. Sheaff, and J. J. Weening. 2007. Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). *Am J Transplant* 7: 518-526.

19. Ozkaynak, E., L. Wang, A. Goodearl, K. McDonald, S. Qin, T. O'Keefe, T. Duong, T. Smith, J.-C. Gutierrez-Ramos, J. B. Rottman, A. J. Coyle, and W. W. Hancock. 2002. Programmed Death-1 targeting can promote allograft survival. *J Immunol* 169: 6546-6553.

20. Kirby, J. A., S. Ikuta, K. Clark, G. Proud, T. W. Lennard, and R. M. Taylor. 1991. Renal allograft rejection: investigation of alloantigen presentation by cultured human renal epithelial cells. *Immunology* 72: 411-417.

21. Obata, F., K. Yoshida, M. Ohkubo, Y. Ikeda, Y. Taoka, Y. Takeuchi, N. Shinohara, T. Endo, and S. Baba. 2005. Contribution of $CD4^+$ and $CD8^+$ T cells and interferon-gamma to the progress of chronic rejection of kidney allografts: the Th1 response mediates both acute and chronic rejection. *Transpl Immunol* 14: 21-25.

22. Ding, H., X. Wu, and W. Gao. 2005. PD-L1 is expressed by human renal tubular epithelial cells and suppresses T cell cytokine synthesis. *Clin Immunol* 115: 184-191.

23. Pfistershammer, K., C. Klauser, W. F. Pickl, J. Stockl, J. Leitner, G. Zlabinger, O. Majdic, and P. Steinberger. 2006. No evidence for dualism in function and receptors: PD-L2/B7-DC is an inhibitory regulator of human T cell activation. *Eur J Immunol* 36: 1104-1113.

24. Tanaka, K., M. J. Albin, X. Yuan, K. Yamaura, A. Habicht, T. Murayama, M. Grimm, A. M. Waaga, T. Ueno, R. F. Padera, H. Yagita, M. Azuma, T. Shin, B. R. Blazar, D. M. Rothstein, M. H. Sayegh, and N. Najafian. 2007. PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. *J Immunol* 179: 5204-5210.

25. Habicht, A., R. Kewalaramani, M. D. Vu, G. Demirci, B. R. Blazar, M. H. Sayegh, and X. C. Li. 2007. Striking Dichotomy of PD-L1 and PD-L2 pathways in regulating alloreactive $CD4^+$ and $CD8^+$ T cells in vivo. *Am J Transplant* 7: 2683-2692.

26. Shen, L., Y. Jin, G. J. Freeman, A. H. Sharpe, and M. R. Dana. 2007. The function of donor versus recipient Programmed Death-Ligand 1 in corneal allograft survival. *J Immunol* 179: 3672-3679.

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References from the introduction

1. Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 4: 336-347.
2. Leonard J. Appleman, V. A. B. 2003. T cell anergy and costimulation. *Immunol Rev* 192: 161-180.
3. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat Rev Immunol* 2: 116-126.
4. Janeway jr., C. A., P. Travers, W. Mark, and S. Mark. 2002. Immunologie. *Berlin Spektrum Akademischer Verlag* 5. Auflage.
5. Kirk, A. D., L. C. Burkly, D. S. Batty, R. E. Baumgartner, J. D. Berning, K. Buchanan, J. H. Fechner, R. L. Germond, R. L. Kampen, N. B. Patterson, S. J. Swanson, D. K. Tadaki, C. N. TenHoor, L. White, S. J. Knechtle, and D. M. Harlan. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med* 5: 686-693.
6. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol* 3: 1097-1101.
7. Vincenti, F., and M. Luggen. 2007. T cell costimulation: a rational target in the therapeutic armamentarium for autoimmune diseases and transplantation. *Annu Rev Med* 58: 347-358.
8. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu Rev Immunol* 23: 515-548.
9. Hofmeyer, K. A., A. Ray, and X. Zang. 2008. The contrasting role of B7-H3. *Proc Natl Acad Sci USA* 105: 10277-10278.
10. Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo J* 11: 3887-3895.
11. Freeman, G. J., A. J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L. J. Fitz, N. Malenkovich, T. Okazaki, M. C. Byrne, H. F. Horton, L. Fouser,

L. Carter, V. Ling, M. R. Bowman, B. M. Carreno, M. Collins, C. R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 192: 1027-1034.

12. Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe, and G. J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2: 261-268.
13. Keir, M. E., M. J. Butte, G. J. Freeman, and A. H. Sharpe. 2008. PD-1 and its ligands in tolerance and immunity. *Ann Rev Immunol* 26: 677-704.
14. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11: 141-151.
15. Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291: 319-322.
16. Sharpe, A. H., E. J. Wherry, R. Ahmed, and G. J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8: 239-245.
17. Holets, L. M., J. S. Hunt, and M. G. Petroff. 2006. Trophoblast CD274 (B7-H1) is differentially expressed across gestation: influence of oxygen concentration. *Biol Reprod* 74: 352-358.
18. Guleria, I., A. Khosroshahi, M. J. Ansari, A. Habicht, M. Azuma, H. Yagita, R. J. Noelle, A. Coyle, A. L. Mellor, S. J. Khoury, and M. H. Sayegh. 2005. A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J Exp Med* 202: 231-237.
19. Reynoso, E. D., K. G. Elpek, L. Francisco, R. Bronson, A. Bellemare-Pelletier, A. H. Sharpe, G. J. Freeman, and S. J. Turley. 2009. Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade. *J Immunol* 182: 2102-2112.

20. Ansari, M. J. I., A. D. Salama, T. Chitnis, R. N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S. J. Khoury, H. Auchincloss, Jr., and M. H. Sayegh. 2003. The Programmed Death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J Exp Med* 198: 63-69.

21. Salama, A. D., T. Chitnis, J. Imitola, M. J. I. Ansari, H. Akiba, F. Tushima, M. Azuma, H. Yagita, M. H. Sayegh, and S. J. Khoury. 2003. Critical role of the Programmed Death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J Exp Med* 198: 71-78.

22. Carter, L. L., M. W. Leach, M. L. Azoitei, J. Cui, J. W. Pelker, J. Jussif, S. Benoit, G. Ireland, D. Luxenberg, G. R. Askew, K. L. Milarski, C. Groves, T. Brown, B. A. Carito, K. Percival, B. M. Carreno, M. Collins, and S. Marusic. 2007. PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 182: 124-134.

23. Brian T. Fife, J. A. B. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol Rev* 224: 166-182.

24. Wang, L., R. Han, and W. W. Hancock. 2007. Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance. *Eur J Immunol* 37: 2983-2990.

25. Ozkaynak, E., L. Wang, A. Goodearl, K. McDonald, S. Qin, T. O'Keefe, T. Duong, T. Smith, J.-C. Gutierrez-Ramos, J. B. Rottman, A. J. Coyle, and W. W. Hancock. 2002. Programmed Death-1 targeting can promote allograft survival. *J Immunol* 169: 6546-6553.

26. Sandner, S. E., M. R. Clarkson, A. D. Salama, A. Sanchez-Fueyo, C. Domenig, A. Habicht, N. Najafian, H. Yagita, M. Azuma, L. A. Turka, and M. H. Sayegh. 2005. Role of the Programmed Death-1 pathway in regulation of alloimmune responses in vivo. *J Immunol* 174: 3408-3415.

27. Koga, N., J.-i. Suzuki, H. Kosuge, G. Haraguchi, Y. Onai, H. Futamatsu, Y. Maejima, R. Gotoh, H. Saiki, F. Tsushima, M. Azuma, and M. Isobe. 2004. Blockade of the interaction between PD-1 and PD-L1 accelerates graft arterial disease in cardiac allografts. *Arterioscler Thromb Vasc Biol* 24: 2057-2062.

28. Keir, M. E., S. C. Liang, I. Guleria, Y. E. Latchman, A. Qipo, L. A. Albacker, M. Koulmanda, G. J. Freeman, M. H. Sayegh, and A. H. Sharpe. 2006. Tissue

expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203: 883-895.

29. Shen, L., Y. Jin, G. J. Freeman, A. H. Sharpe, and M. R. Dana. 2007. The function of donor versus recipient Programmed Death-Ligand 1 in corneal allograft survival. *J Immunol* 179: 3672-3679.
30. Blazar, B. R., B. M. Carreno, A. Panoskaltsis-Mortari, L. Carter, Y. Iwai, H. Yagita, H. Nishimura, and P. A. Taylor. 2003. Blockade of Programmed Death-1 engagement accelerates graft-versus-host disease lethality by an IFN-γ-dependent mechanism. *J Immunol* 171: 1272-1277.
31. Schuchmann, M., R. G. Meyer, E. Distler, E. von Stebut, J. Kuball, E. Schnurer, T. Wolfel, M. Theobald, A. Konur, S. Gregor, O. Schreiner, C. Huber, P. R. Galle, G. Otto, and W. Herr. 2008. The programmed death (PD)-1/PD-ligand 1 pathway regulates graft-versus-host-reactive CD8 T cells after liver transplantation. *Am J Transplant* 8: 2434-2444.
32. Okazaki, T., and T. Honjo. 2007. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol* 19: 813-824.
33. Gao, Q., X. Y. Wang, S. J. Qiu, I. Yamato, M. Sho, Y. Nakajima, J. Zhou, B. Z. Li, Y. H. Shi, Y. S. Xiao, Y. Xu, and J. Fan. 2009. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. *Clin Cancer Res* 15: 971-979.
34. Blank, C., and A. Mackensen. 2007. Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. *Cancer Immunol Immunother* 56: 739-745.
35. Thompson, R. H., M. D. Gillett, J. C. Cheville, C. M. Lohse, H. Dong, W. S. Webster, K. G. Krejci, J. R. Lobo, S. Sengupta, L. Chen, H. Zincke, M. L. Blute, S. E. Strome, B. C. Leibovich, and E. D. Kwon. 2004. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci USA* 101: 17174-17179.
36. Thompson, R. H., H. Dong, C. M. Lohse, B. C. Leibovich, M. L. Blute, J. C. Cheville, and E. D. Kwon. 2007. PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. *Clin Cancer Res* 13: 1757-1761.
37. Parsa, A. T., J. S. Waldron, A. Panner, C. A. Crane, I. F. Parney, J. J. Barry, K. E. Cachola, J. C. Murray, T. Tihan, M. C. Jensen, P. S. Mischel, D. Stokoe,

and R. O. Pieper. 2007. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 13: 84-88.

38. Iwai, Y., S. Terawaki, M. Ikegawa, T. Okazaki, and T. Honjo. 2003. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med* 198: 39-50.
39. Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682-687.
40. Day, C. L., D. E. Kaufmann, P. Kiepiela, J. A. Brown, E. S. Moodley, S. Reddy, E. W. Mackey, J. D. Miller, A. J. Leslie, C. DePierres, Z. Mncube, J. Duraiswamy, B. Zhu, Q. Eichbaum, M. Altfeld, E. J. Wherry, H. M. Coovadia, P. J. R. Goulder, P. Klenerman, R. Ahmed, G. J. Freeman, and B. D. Walker. 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443: 350-354.
41. Urbani, S., B. Amadei, D. Tola, M. Massari, S. Schivazappa, G. Missale, and C. Ferrari. 2006. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. *J Virol* 80: 11398-11403.
42. Geng, L., G. Jiang, Y. Fang, S. Dong, H. Xie, Y. Chen, M. Shen, and S. Zheng. 2006. B7-H1 expression is upregulated in peripheral blood $CD14^+$ monocytes of patients with chronic hepatitis B virus infection, which correlates with higher serum IL-10 levels. *J Viral Hep* 13: 725-733.
43. Trabattoni, D., M. Saresella, M. Biasin, A. Boasso, L. Piacentini, P. Ferrante, H. Dong, R. Maserati, G. M. Shearer, L. Chen, and M. Clerici. 2003. B7-H1 is up-regulated in HIV infection and is a novel surrogate marker of disease progression. *Blood* 101: 2514-2520.
44. Butte, M. J., M. E. Keir, T. B. Phamduy, A. H. Sharpe, and G. J. Freeman. 2007. Programmed Death-1 Ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 27: 111-122.
45. Butte, M. J., V. Peña-Cruz, M.-J. Kim, G. J. Freeman, and A. H. Sharpe. 2008. Interaction of human PD-L1 and B7-1. *Mol Immunol* 45: 3567-3572.
46. Azuma, T., S. Yao, G. Zhu, A. S. Flies, S. J. Flies, and L. Chen. 2008. B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells. *Blood* 111: 3635-3643.
47. Berger, R., R. Rotem-Yehudar, G. Slama, S. Landes, A. Kneller, M. Leiba, M. Koren-Michowitz, A. Shimoni, and A. Nagler. 2008. Phase I safety and

pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies. *Clin Cancer Res* 14: 3044-3051.

48. Wong, R. M., R. R. Scotland, R. L. Lau, C. Wang, A. J. Korman, W. M. Kast, and J. S. Weber. 2007. Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. *Int Immunol* 19: 1223-1234.
49. Schreiner, B., M. Mitsdoerffer, B. C. Kieseier, L. Chen, H.-P. Hartung, M. Weller, and H. Wiendl. 2004. Interferon-β enhances monocyte and dendritic cell expression of B7-H1 (PD-L1), a strong inhibitor of autologous T-cell activation: relevance for the immune modulatory effect in multiple sclerosis. *J Neuroimmunol* 155: 172-182.
50. Wiesemann, E., M. Deb, C. Trebst, B. Hemmer, M. Stangel, and A. Windhagen. 2008. Effects of interferon-β on co-signaling molecules: upregulation of CD40, CD86 and PD-L2 on monocytes in relation to clinical response to interferon-β treatment in patients with multiple sclerosis. *Multiple Sclerosis* 14: 166-176.
51. Rastegar, A., and M. Kashgarian. 1998. The clinical spectrum of tubulointerstitial nephritis. *Kidney Int* 54: 313-327.
52. Cornell, L. D., R. N. Smith, and R. B. Colvin. 2008. Kidney transplantation: mechanisms of rejection and acceptance. *Annu Rev Pathol* 3: 189-220.
53. Murashima, M., J. Tomaszewski, and J. D. Glickman. 2007. Chronic tubulointerstitial nephritis presenting as multiple renal nodules and pancreatic insufficiency. *Am J Kidney Dis* 49: e7-e10.
54. Barry, M., and R. C. Bleackley. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2: 401-409.
55. Neilson, E. G. 1993. The nephritogenic T lymphocyte response in interstitial nephritis. *Semin Nephrol* 13: 496-502.
56. Spanou, Z., M. Keller, M. Britschgi, N. Yawalkar, T. Fehr, J. Neuweiler, M. Gugger, M. Mohaupt, and W. J. Pichler. 2006. Involvement of drug-specific T cells in acute drug-induced interstitial nephritis. *J Am Soc Nephrol* 17: 2919-2927.
57. Nguan, C. Y. C., and C. Du. 2009. Renal tubular epithelial cells as immunoregulatory cells in renal allograft rejection. *Transplant Rev* in press.

58. van Kooten, C., and M. R. Daha. 2001. Cytokine cross-talk between tubular epithelial cells and interstitial immunocompetent cells. *Curr Opin Nephrol Hypertens* 10: 55-59.

59. Wüthrich, R., L. Glimcher, M. Yui, A. Jevnikar, S. Dumas, and V. Kelley. 1990. MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. *Kidney Int* 37: 783-792.

60. Rubin-Kelley, V. E., and A. M. Jevnikar. 1991. Antigen presentation by renal tubular epithelial cells. *J Am Soc Nephrol* 2: 13-26.

61. Wahl, P., R. Schoop, G. Bilic, J. Neuweiler, M. Le Hir, S. K. Yoshinaga, and R. P. Wüthrich. 2002. Renal Tubular Epithelial Expression of the Costimulatory Molecule B7RP-1 (Inducible Costimulator Ligand). *J Am Soc Nephrol* 13: 1517-1526.

62. Wahl, P., and R. P. Wüthrich. 2004. Role of the B7RP-1/ICOS pathway in renal tubular epithelial antigen presentation to $CD4^+$ Th1 and Th2 cells. *Nephron Exp Nephrol* 98: e31-38.

63. Lutz, J., R. Lu, M. Strobl, H. Huang, M. Deng, M. Wang, N. Ouyang, and U. Heemann. 2007. ICOS/B7RP-1 interference in mouse kidney transplantation. *Transplantation* 84: 223-230

64. Odobasic, D., A. R. Kitching, T. J. Semple, and S. R. Holdsworth. 2006. Inducible Co-Stimulatory Molecule Ligand is protective during the induction and effector phases of crescentic glomerulonephritis. *J Am Soc Nephrol* 17: 1044-1053.

65. Chen, Y., J. Zhang, J. Li, L. Zou, T. Zhao, Y. Tang, and Y. Wu. 2006. Expression of B7-H1 in inflammatory renal tubular epithelial cells. *Nephron Exp Nephrol* 102: e81-92.

66. de Haij, S., A. M. Woltman, L. A. Trouw, A. C. Bakker, S. W. Kamerling, S. W. van der Kooij, L. Chen, R. A. Kroczek, M. R. Daha, and C. van Kooten. 2005. Renal tubular epithelial cells modulate T-cell responses via ICOS-L and B7-H1. *Kidney Int* 68: 2091-2102.

67. Schoop, R., P. Wahl, M. Le Hir, U. Heemann, M. Wang, and R. P. Wüthrich. 2004. Suppressed T-cell activation by IFN-γ-induced expression of PD-L1 on renal tubular epithelial cells. *Nephrol Dial Transplant* 19: 2713-2720.

LIST OF PUBLICATIONS

These publications contributed to the dissertation

- Waeckerle-Men Y, [1]Starke A, Wahl PR, Wüthrich RP: *Limited costimulatory molecule expression on renal tubular epithelial cells impairs T cell activation.* Kidney Blood Press Res 2007;30:421-429.
    - [1] involved in performance and analysis of several experiments (preparation of primary TECs and OT-1 $CD8^+$ T cells, part of the FACS analysis) and involved in writing the manuscript

- Waeckerle-Men Y, [2]Starke A, Wüthrich RP: *PD-L1 partially protects renal tubular epithelial cells from the attack of $CD8^+$ cytotoxic T cells*. Nephrol. Dial. Transplant. 2007;22:1527-1536.
    - [2]involved in performing and analyzing several experiments (preparation of primary TECs and OT-1 $CD8^+$ T cells, part of the FACS analysis) and participated in writing the manuscript

- [3]Starke A, Wüthrich RP, Waeckerle-Men Y: *TGF-Beta treatment modulates PD-L1 and CD40 expression in proximal renal tubular epithelial cells and enhances $CD8^+$ cytotoxic T-cell responses*. Nephron Exp Nephrol 2007;107:e22-e29.
    - [3]performance of all experiments; data analysis; design and writing of the complete manuscript

- [4]Astrid Starke, Stephan Segerer, Michel Le Hir, Miyuki Azuma, Thomas Fehr, Rudolf P. Wüthrich and Ying Waeckerle-Men: *Establishment of an antigen-specific model of tubulointerstitial nephritis*. Manuscript in preparation.
    - [4]performance of experiments; data analysis; design and writing of the manuscript

- [5]Starke A, Lindenmeyer MT, Segerer S, Neusser M, Schmid DM, Cohen CD, Fehr T, Wüthrich RP, Waeckerle-Men Y. *Renal tubular PD-L1 suppresses alloreactive human T cell responses*. Submitted
    - [5]performance and data analysis of the *in vitro* experiments (Figures 1-4); design and writing of the complete manuscript

Other publications during the time as PhD student:

- Yung GP, Valli PV, Starke A, Mueller RJ, Fehr T, Cesar-Ozpamir M, Schanz U, Weber M, Wüthrich RP, Seebach JD, Stussi G: *Flow cytometric measurement of ABO antibodies in ABO-incompatible living donor kidney transplantation*. Transplantation 2007;84:S20-23.

- Wahl PR, Le Hir M, Vogetseder A, Arcaro A, Starke A, Waeckerle-Men Y, Serra AL, Wüthrich RP: *Mitotic activation of Akt signalling pathway in Han:SPRD rats with polycystic kidney disease*. Nephrology (Carlton) 2007;12:357-363.

- Serra AL, Braun SC, Starke A, Savoca R, Hersberger M, Russmann S, Corti N, Wüthrich RP: *Pharmacokinetics and pharmacodynamics of cinacalcet in patients with hyperparathyroidism after renal transplantation.* Am J Transplant. 2008;4:803-10.

CURRICULUM VITAE

PERSONAL DATA

Last name:	Starke
First name:	Astrid
Date of Birth:	1st April, 1982
Place of Birth:	Rostock, Germany
Nationality:	German

EDUCATION

07/2000	Abitur at Friderico Francisceum, Bad Doberan, Germany
10/2000-10/2005	Study of Human Biology at the Ernst-Moritz-Arndt University Greifswald, Germany, grade: very good (1,5) degree: Diplom Humanbiologin
09/2002	"Vordiplom", exams in chemistry, human anatomy and physiology, biochemistry, microbiology, mathematics
02-03/2004	Internship at the Department of Dermatology, Allergy Unit, University Hospital Zürich, Switzerland; involved in a dissertation about birch pollen-related food allergy
09/2004	"Diplomsprüfungen" in immunology (major), medical biochemistry, virology
10/2004-09/2005	Diploma thesis: "The influence of acute stress on the immune response" in the group of Prof. Christine Schütt, Department of Immunology, University of Greifswald grade: very good (1,3)

01/2006-06/2009	Dissertation in the PhD program "Integrative Molecular Medicine (imMed)" at the Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zürich and Division of Nephrology, University Hospital Zürich, Switzerland in the group of Prof. Rudolf P. Wüthrich and PD Dr. Ying Wäckerle-Men Topic: Role of the PD-1/PD-L1 co-inhibitory pathway in immune-mediated renal injury

AWARDS

2006 and 2008	Travel grants from "Schweizerische Nierenstiftung" for the attendance of the renal week of the American Society of Nephrology (ASN), San Diego, USA and the American Transplant Congress (ATC), Toronto, CA

EXTRACURRICULAR ACTIVITIES

06/2004	courses in business start-up and entrepreneurship; establishment and succession of a business organized by the faculty of economics, University Greifswald, Germany
11/2006	"Embracing Life-Challenges and Questions in Patenting for Life Sciences" organised by the Forum for Genetic Research of the Swiss Academy of Sciences

COLLEGIATE ACTIVITIES

2001-2005	Member of Humanbiologie Greifswald e.V. and activity in the subgroup puls ("Public understanding of Life Science")
06/2002	Management of a workshop about "Aids as global problem", GrIStuF e.V. (Greifswald International Student Festival)

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